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THE JOURNAL OF GENERAL PHYSIOLOGY

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JACQUES LOEB W. J. V. OSTERHOUT

VOLUME I, NO. 1 SEPTEMBER, 1918

PUBLISHED BIMONTHLY
AT 2419-21 GREENMOUNT AVE., BALTIMORE, MD.
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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Application for entry as second-class matter at the Post Office at Baltimore, Md., under the Act of March 3, 1879.

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The Journal of Biological Chemistry is issued monthly, and since its foundation in 1905 thirty-five volumes of approximately 550 pages each have been completed. The subscription price is $3.00 per volume to subscribers in the United States and Canada; $3.25 to those in other countries. The number of volumes issued yearly averages four.

THE JOURNAL OF GENERAL PHYSIOLOGY

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The Journal of General Physiology is published by The Rockefeller Institute for Medical Research and is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter. Information as to contributions and subscriptions is given in full on the back cover.

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ON THE DYNAMICS OF PHOTOSYNTHESIS.

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(Received for publication, July 8, 1918.)

Although a great deal of attention has been paid to photosynthesis, nothing is known of the dynamics of the process. This aspect of the matter especially deserves investigation as furnishing a new point of attack upon this difficult problem.

We cannot analyze the dynamics of photosynthesis without first securing accurate data. A preliminary difficulty lies in the control of temperature; when leaves of land plants are exposed to sunlight, changes of temperature at once take place in the leaf and it is found that even under favorable conditions of control the temperature of the leaf may fluctuate as much as 10°C. in a half hour period. To avoid this difficulty, the writers have employed certain aquatic plants, which form thin layers or filaments, whose temperature can be regulated to a sufficient extent for the purposes of the investigation.

The fronds of the marine alga, *Ulva rigida* (sea lettuce), are so useful for this purpose that most of the experimental work was confined to them, although other material was used for comparison. These fronds consist of only two layers of cells and are so thin (about 0.078 mm.) that their temperature remains very close to that of the surrounding liquid. A further advantage of thin fronds is that gaseous exchange is extremely rapid.

The experiments on *Ulva* were carried on at the Marine Biological Laboratory at Woods Hole during the month of August when an abundant supply of excellent material was available.

To obtain data for the study of dynamics it is necessary to determine at frequent intervals how much photosynthesis has taken place. None of the available methods was satisfactory for our purpose. The method of counting bubbles is open to serious objections while the method of analyzing the gases in solution, as developed by Black-

man and Smith,[1] is not sufficiently accurate and convenient. The difficulty was solved by developing a method which depends on the fact that as the plants abstract carbon dioxide from the solution it becomes more alkaline.

In collaboration with Loeb[2] one of the authors had observed that certain marine algæ when exposed to sunlight cause the sea water to become more alkaline. Similar observations had been previously made by various observers[3] upon fresh water plants in solutions containing bicarbonates. If bicarbonates are absent, little or no effect is observed. The greater degree of alkalinity produced in the presence of bicarbonates is due to the fact that the plants abstract CO_2 from bicarbonates. Thus in sea water (which normally contains carbonates and bicarbonates) the alkalinity produced in this way may amount to more than pH = 9.

In the case of marine plants it is not necessary to add bicarbonates, since the sea water contains a sufficient amount. Such plants can therefore be studied in their natural environment, which is a distinct advantage over the methods hitherto employed, in which concentrations of CO_2 greatly in excess of the normal were maintained during the experiments.

In this connection it may be mentioned that some authors state that the concentration of free CO_2 is about the same in solutions containing carbonates and bicarbonates as in the air above and that plants in such solutions have no more CO_2 at their disposal than land plants. Aside from the fact that the amount of free CO_2 in sea water is not known, they seem to overlook the fact that when free CO_2 is abstracted from a solution of carbonates and bicarbonates it is at once partially replaced as the result of the dissociation of the carbonates and bicarbonates, so that the plant receives at once what otherwise must be more slowly supplied by diffusion. The carbonates and bicarbonates constitute a reservoir of CO_2 which may be depleted by photosynthesis during the day and filled up during the night by

[1] Blackman, F. F., and Smith, A. M., *Proc. Roy. Soc., Series B*, 1911, lxxxiii, 374.

[2] Loeb, J., The dynamics of living matter, New York, 1906, 98. Cf. Moore, B., Prideaux, E. B. R., and Herdman, G. A., *Proc. and Tr. Liverpool Biol. Soc.*, 1915, xxix, 233.

[3] Czapek, F., Biochemie der Pflanzen, Jena, 2te Aufl., 1913, i, 519.

diffusion from the air. The amount of CO_2 at the immediate disposal of the plant therefore depends largely on the amount of carbonates and bicarbonates present.

The usefulness of carbonates and bicarbonates in this connection is greatly increased by the fact that the plants are able to split them so as to extract from them much more CO_2 than can be removed by bubbling a stream of hydrogen through the solution.

In order to measure the degree of alkalinity produced by *Ulva*, a piece of the frond was placed in a tube of Pyrex glass[4] (about 12 mm. in diameter and about 5 cm. long) in such a manner that it completely covered the inside of the tube for the greater portion of its length. Fronds were chosen which were sufficiently stiff, so that their own elasticity caused them to remain pressed against the inner surface of the glass tube even when liquid was poured in and out or shaken back and forth in the tube.

The glass tube was sealed off at one end, while at the other it was furnished with a short piece of rubber tubing covered with paraffin.[5] The covering of paraffin was continuous and care was taken to renew it frequently.

After placing the frond in the tube, the latter was filled with sea water containing indicator[6] and the rubber tube was clamped shut. In some cases a small bubble of air was left in the tube to act as a stirrer; in other cases the tube was completely filled with sea water and the stirring was effected by a small piece of paraffin or by a glass bead covered with paraffin.

In order to determine the degree of alkalinity produced by photosynthesis two methods were used. In the first, the indicator was added to the sea water containing *Ulva* after a definite exposure to

[4] This glass was chosen because it does not give off measurable quantities of alkali during the period of the experiment.

[5] It is necessary to use paraffin which will not give off measurable quantities of acid during the time of the experiment. For this purpose paraffin of a high melting point is usually advantageous. Rubber should be used which gives off the minimum amount of acid; the rubber used in these experiments was repeatedly boiled before using.

[6] Ten drops of saturated alcoholic phenolphthalein was added to 1 liter of sea water. This makes the concentration of alcohol 0.0067 M and that of phenolphthalein 0.0001 M.

sunlight; in the second, the indicator was added to the sea water before the exposure began. In the latter case there was a possibility that the presence of the indicator might affect the amount of photosynthesis but it was found by control experiments that this was not the case with the concentrations employed in these experiments.

There is an advantage in adding the indicator at the start since this permits us to compare the times required to produce a given amount of change under different conditions. A comparison of the values thus obtained is more valuable than a comparison of the amounts of CO_2 abstracted during equal times, for the former procedure compares the reaction velocities accurately while the latter may not. In case the indicator is added at the end, instead of at the beginning, curves may be constructed (plotting CO_2 against time) from which the time required for equal changes in alkalinity may be obtained by interpolation.

In case any substance is added to the solution which changes its buffer value, due allowance must be made for this fact. An apparatus for determining the buffer action of added reagents has recently been described by one of us.[7]

It was found by preliminary experiments that the amount of CO_2 abstracted by the plant was an approximately linear function of the pH value (in the range here employed, between pH 8.1 and pH 8.3).

It was necessary to ascertain whether the degree of alkalinity produced was a reliable measure of the amount of photosynthesis. This was done by making simultaneous determinations of the degree of alkalinity and the amount of oxygen evolved (by a modification of Winkler's method recently described by us[8]). The results show that the amount of photosynthesis, as indicated by the evolution of oxygen, is approximately a linear function (in this range) of the change in the pH value of the sea water. This being so we can measure the amount of photosynthesis by determining the change in pH value regardless of any possible complications, such as excretion of alkali by the plant.

Since the plants produce CO_2 by respiration this must be taken into consideration. Experiments conducted under precisely the same conditions, except that light was excluded, showed that the respiration was

[7] Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxv, 237.

[8] Osterhout, W. J. V., and Haas, A. R. C., *J. Biol. Chem.*, 1917, xxxii, 141.

practically constant. It is, therefore, easy to make a correction for it. It does not affect the form of the curve of photosynthesis found in the present investigation. That no acid other than carbonic is given off by the plant is shown by experiments in the dark in which the acidity produced was completely removed by a stream of hydrogen.

In order to ascertain how much photosynthesis had taken place after a definite time the pink color produced by the *Ulva* was matched against the colors of a series of Pyrex glass tubes[9] (of the same size) containing the same concentration of indicator in a series of buffer solutions of known alkalinity.[10] The matching was done under a "Daylight" lamp, which is invaluable for this purpose.

In this way the degree of alkalinity produced may be easily ascertained and since this corresponds to the amount of oxygen evolved, it gives us a direct measure of photosynthesis, provided we know the amount of CO_2 corresponding to the observed changes in alkalinity. This may be determined by the method referred to above.[7]

In order to carry out such investigations as the present one or to study the effects of temperature, light intensity, etc., it is not necessary to know the amount of CO_2 abstracted; it is sufficient to compare the time required to produce the same change in the color of the indicator under different conditions.

The experimental procedure was as follows: Young, vigorous plants of *Ulva* (deep green in color and not over 4 inches in diameter) were collected in the afternoon and placed in running sea water in the laboratory. In the evening they were covered with a dark screen so that the morning light could not reach them. On the following morning the plants were placed in a water bath at 27°C. and allowed to come to the temperature of the bath.

[9] These tubes were prepared and clamped shut in precisely the same manner as the tubes containing *Ulva*.

[10] Cf. Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131; *Ergebn. Physiol.*, 1912, xii, 393. Hoeber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 4th edition, 1914, 169. Bayliss, W. M., Principles of general physiology, New York, 1915, 203.

For the pH values needed in these investigations mixtures of 0.05 M borax and 0.2 M boric acid (to each liter of boric acid 2.925 gm. NaCl is added) are useful. The following table gives the pH values of a series of mixtures (Palitzsch, S., *Biochem. Z.*, 1915, lxx, 333; *Compt. rend. lab. Carlsberg*, 1916, xi, 199). Cf.

The experiment was started in the following manner. When everything (plants, sea water, and tubes) had come to the temperature of the bath, the plants were placed in the tubes, the rubber tubes were clamped shut, and the tubes were then placed in the bath so that they were about half an inch below the surface of the water, making an angle of about 20° with the surface, and exposed to direct sunlight.

Under these circumstances the alga receives abundant sunlight for photosynthesis. The amount of sunlight is affected by reflection from the surface of the water and the amount of reflection is influenced by the movement of the water due to stirring. But this is a fairly constant factor and of negligible importance. The effect of stirring can be obviated by placing a sheet of glass in contact with

McClendon, J. F., Gault, C. E., and Mulholland, S., *Carnegie Institution of Washington, Publication 251*, 1917, 21.

0.2 M boric acid.	0.05 M borax.	pH
cc.	*cc.*	
0	10	9.24
1.0	9.0	9.11
2.0	8.0	8.98
3.0	7.0	8.84
4.0	6.0	8.69
4.5	5.5	8.60
5.0	5.0	8.51
5.5	4.5	8.41
6.0	4.0	8.31
6.5	3.5	8.20
7.0	3.0	8.08
7.5	2.5	7.94
7.7	2.3	7.88
8.0	2.0	7.78
8.5	1.5	7.60
9.0	1.0	7.36
9.4	0.6	7.09
9.7	0.3	6.77

By plotting the cc. of borax as ordinates and the pH values as abscissæ a curve is obtained from which intermediate values can be obtained by graphic interpolation. From the pH values found in sea water 0.21 must be subtracted on account of the "salt error." In the present investigation these values were carefully checked by means of the hydrogen electrode.

the surface so as to eliminate ripples, or by using in place of the tub a tank with a vertical glass wall against which the tube is held in such a way that the light falls upon the tube after passing through the glass wall.

The temperature of the bath was kept constant within 1°C. There was no need of more accurate control of temperature under the conditions of the experiment. A thermometer was inserted into the tube in many of the experiments.[11] On exposure to sunlight the temperature of the tube rose and remained slightly above that of the bath, but under the conditions of the experiment the difference was almost constant. Since the tissues used were so extremely thin, it is safe to assume that the temperature of the tissues was practically the same as that of the liquid in the tube.

The tube containing *Ulva* was allowed to remain in sunlight, with occasional shaking to stir its contents, until a standard shade of pink was produced which matched that of a selected buffer solution (for purposes of matching a clear space was left in the tube above or below the frond). When the color of the tube was seen to be closely approaching that of the selected buffer solution, the tube was removed for an instant from the bath, shaken, and placed beside the selected buffer solution under the "Daylight" lamp. If the tint was not as deep as that of the buffer, the tube was returned to the bath. As soon as the desired tint was reached the time was noted, the sea water was emptied from the tube, and a fresh sample of sea water (containing indicator) was poured in. The tube was again exposed to sunlight and the time required to produce the same tint was noted. This procedure was repeated as often as necessary.

The experiments were carried out on cloudless days during the month of August. In case clouds interfered with the sunlight at any time during the course of the experiment the whole experiment was rejected. At first it was feared that the increase in the intensity of the sunlight during the morning and its decline during the afternoon might affect the results. In order to ascertain whether this was the case the experiments were started at various times during the

[11] The glass of the thermometer did not give off sufficient alkali to affect the results.

day. It was found that if the work was done between 9 a.m. and 4 p.m. the changes in the intensity of the sunlight might be neglected.

The results obtained are given in Table I, which represents the average of five experiments, and are illustrated in Fig. 1.

It is evident from Fig. 1 that the rate increases rapidly at first, then more and more slowly until it finally ceases to increase. From this point onward a steady rate is maintained.[12] The result is surprising, but in view of the fact that it has been confirmed by numerous experiments with *Ulva* as well as by experiments on *Enteromorpha*, *Spirogyra*, *Hydrodictyon*, *Potamogeton*, and other plants[13] it seems to be well established.

TABLE I.

Period.	Time required to produce standard alkalinity.	Total time exposed.	Amount of photosynthesis.	
			Observed.	Calculated.
	min.	*min.*		
1	35.7	35.7	1	0.92
2	25.9	61.6	2	2.07
3	23.3	84.9	3	3.18
4	21.7	106.6	4	4.23
5	20.4	127.0	5	5.23
6	20.3	147.3	6	6.22
7	20.5	167.8	7	7.22

Average of five experiments at 27° ± 0.5°C.

Two questions of great interest now present themselves. First, why does the rate increase at the start?[14] Second, why does it finally become stationary?

[12] This steady rate is not the same for each piece of frond but each piece is fairly constant in its rate, so that if the material is kept over night it will be found the next morning that different pieces may start out at different rates, but the steady rate which each piece finally attains is practically the same as the steady rate which the same piece had the day before. In fact it is possible to recognize the various pieces by this means.

[13] In experiments on fresh water algæ a small amount of sodium bicarbonate was added to the water.

[14] This acceleration is not due to the increase in the intensity of light as the sun gets higher for it was also observed when the experiments were started at noon.

The suggestion which first offers itself is that photosynthesis belongs to the class of autocatalytic processes, in which the reaction is catalyzed by one of its own products. Such reactions begin slowly but as more of the catalyzing substance is produced the reaction goes on at an increasingly rapid rate until it begins to slow down as the reacting substances are used up. If these substances are constantly renewed, the reaction will not slow down but continue to go on more and more rapidly.

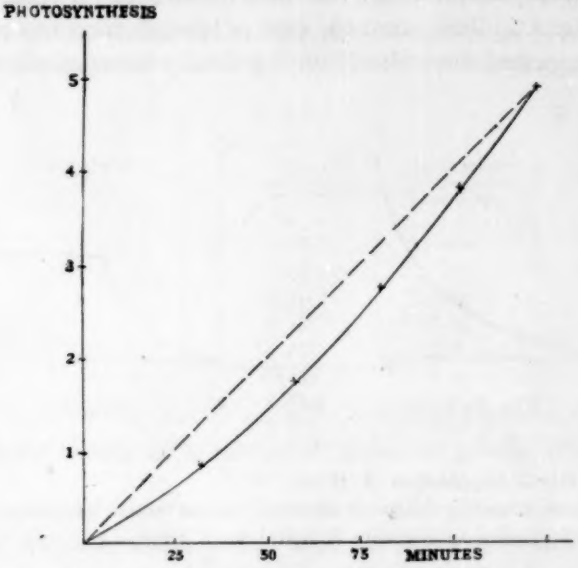

FIG. 1. Curve showing that where *Ulva* is exposed to light the speed of photosynthesis increases until a steady rate is attained. The dotted line expresses a uniform rate.

In our experiments on photosynthesis the reacting substances are constantly renewed.[15] The substances entering into the reaction are presumably carbon dioxide and water. The concentration of the water remains constant, while as soon as the concentration of the carbon dioxide has diminished by a very small amount it is brought back to the original point by the renewal of the sea water.

[15] When the sea water is not changed during the experiment the curve rises more rapidly at first, then bends over to the right as the supply of CO_2 is used up.

If photosynthesis were an autocatalytic reaction, the amount of catalyzer should increase in the manner indicated in Fig. 2 and, under these conditions, the process should continue to increase in speed as time goes on. As a matter of fact it soon attains a steady rate. This might be accounted for by supposing that the concentration of the catalyst cannot exceed a certain amount, being limited by its own solubility. But in that case the rate would increase more and more rapidly up to a certain point and suddenly become stationary when the limit of solubility was reached, the curve of the catalyst being like that shown in Fig. 3.[16] This is not the case. The rate increases rapidly at first then more and more slowly until it finally becomes stationary.

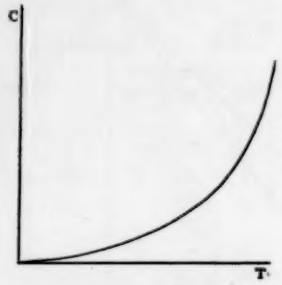

FIG. 2.

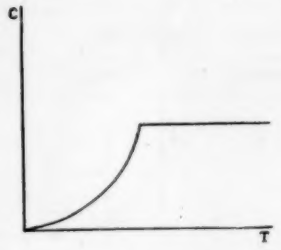

FIG. 3.

FIG. 2. Curve showing increase in the amount of the catalyst when the reaction is autocatalytic (C, catalyst; T, time).

FIG. 3. Curve showing behavior of the catalyst when its concentration is limited by such a factor as solubility (C, catalyst; T, time).

It might be supposed that the speed of the reaction is checked by the accumulation of the products of the reaction. In that case, however, the rate would not become constant but would gradually diminish to zero. Such influence of the products would be possible only in the case of a reversible reaction and we have no ground for believing that photosynthesis comes under this head.[17]

[16] This is because the catalyst from the moment of its production is in solution. It is not analogous to a solid going into solution, which dissolves more slowly as the limit of solubility is approached.

[17] While respiration is in a sense the opposite of photosynthesis the steps in the process are apparently quite different from those found in photosynthesis.

It might also be suggested that the rate becomes constant through the operation of a "limiting factor" such as lack of light, carbon dioxide, or of temperature. But it is evident that the effect of such a factor would be fully felt at the very start of the reaction and that it could not cause a gradual falling off in the increase of speed.

This puts clearly before us a fundamental difficulty. The fact that the rate increases most rapidly at first and then more slowly shows that photosynthesis is not an autocatalytic reaction in the usual sense of the word, for in such a reaction[18] the rate would increase slowly at first, then more and more rapidly as time goes on. We must therefore conclude that photosynthesis belongs in a different category.

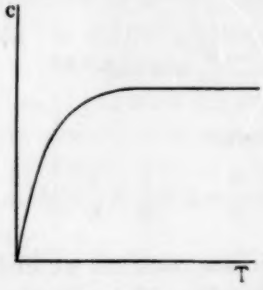

FIG. 4. Curve showing the behavior of the catalyst on the assumption that it is produced by the monomolecular reaction $A \rightarrow C$ (C, catalyst; T, time).

The key to the situation is furnished by the figures in the second column of Table I, which show that if the reaction is catalyzed by a substance, it must be produced more rapidly at first and then more and more slowly. It is also evident that this substance must be limited in amount and that when its production ceases the rate of photosynthesis becomes constant. We may assume that the rate of photosynthesis is proportional to the amount of the catalyst, which we will call C. The figures suggest that this substance may be produced in the manner characteristic of a monomolecular reaction as shown in Fig. 4. We may therefore assume that C is produced by a substance A, under the influence of sunlight, according to the monomolecular reaction: $A \rightarrow C$.

[18] *I.e.*, under the conditions of the present experiment, where the reacting substances are kept approximately constant in composition.

We may now test this assumption by calculating the amount of photosynthesis which is to be expected after the lapse of a given time.

According to the ordinary equation for a monomolecular reaction,

$$C = A - Ae^{-KT}$$

in which T is time, e is the basis of natural logarithms, and K is the velocity constant of the reaction.

We may denote the amount of photosynthesis by P. If the rate of photosynthesis is directly proportional to the amount of C, we may, for convenience, put

$$\frac{dP}{dT} = C;$$

hence

$$\frac{dP}{dT} = A - Ae^{-KT}.$$

On integration this becomes

$$\frac{P}{A} = T - \frac{1}{K} + \frac{1}{K}e^{-KT}.$$

When the rate has become constant we find that a unit amount of photosynthesis is produced in 20.4 minutes (average of the last 3 periods in Table I), hence the rate of photosynthesis at that time is $1 \div 20.4 = 0.049$. This is by assumption equal to C when A is completely transformed into C and this is in turn equal to A at the beginning of the reaction. Hence A at the start $= 0.049$. We may substitute this value in the equation and find the value of K by trial. If we put $K = 0.049$ we get the values given in Table I. Better agreement with the observed values is obtained by taking lower values of K. This produces a gradual falling off in subsequent values, but it is possible that this might actually occur if the experiment could be continued for a sufficient length of time.

The agreement between the observed and the calculated values is very satisfactory except at the start. In this connection it may be pointed out that at the beginning of a reaction disturbances are to be expected.

It is therefore evident that the assumption justifies itself by giving an adequate quantitative explanation of the observed results. The

question then arises whether it is a natural one. It would seem very probable that the light produces a substance which accelerates the reaction and unless this substance is produced in unlimited amount there must come a time when the rate will become steady (or fall off). The assumption therefore seems to be reasonable.

It is attractive to form a hypothesis as to the nature of the catalyst. One might be tempted to suppose that it is chlorophyll but for the fact that some plants which are deep green may not photosynthesize as rapidly as those which possess less chlorophyll.[19] It is of course possible that the less active plants are deficient in some essential factor other than chlorophyll. On the other hand it may be necessary for chlorophyll to be transformed by the light from an inactive into an active form,[20] so that the rate of photosynthesis depends on the amount of "active chlorophyll" present. This would be analogous to the well known activation of enzymes by various means.

An equally satisfactory quantitative explanation is obtained if we suppose the amount of photosynthesis to correspond to the amount of a substance P, produced (under the influence of light) by the monomolecular reactions

$$S \rightarrow M \rightarrow P,$$

in which S represents a constant source (*i.e.*, a substance which does not appreciably diminish during the experiment).

Let us suppose that in the morning, before the frond is exposed to the light, S alone is present. On exposure to light the formation of M and P begins. The amount of M will then increase until it reaches a constant value (when its rate of formation is equal to its rate of decomposition) but the value of P will continually increase, since it does not undergo decomposition. When M has reached a constant value we find (putting K as the velocity constant of the reaction $M \rightarrow P$) that the amount of M decomposed in 1 minute (unit time) is KM; this is also the amount of P which is formed in 1 minute, and since the reaction $S \rightarrow M$ produces just enough of M to balance the loss of M (by transformation into P) the amount of M produced each minute

[19] Aquatic plants taken directly from ice-covered ponds in winter are found to possess but feeble photosynthetic power, though of a deep green color.

[20] Such activation of substances by light is well known in photochemistry.

is KM. Hence if we start in the morning with S alone there will be produced each minute KM and all of this will be transformed into P except what is present at any moment as M. Hence the amount of P produced in the time T is $KMT - M$.

When M has attained its constant value, we may, for convenience, put $M = 1$. The rate of increase of P is then constant and we find from the table that it takes 20.4 minutes to produce one unit of photo-

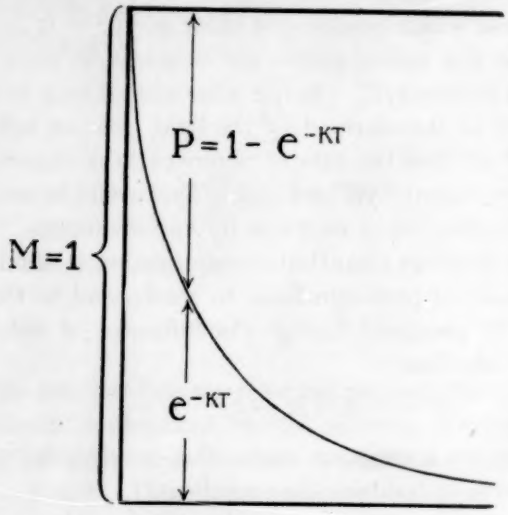

FIG. 5. Curve to illustrate that, as M decomposes in monomolecular fashion to form P, the amount of M left at any given time, T, is e^{-KT} and the amount of P is $1-e^{-KT}$. The abscissæ represent time; the ordinates the amount of M. At the start of the reaction $M=1$.

synthesis; hence $KMT = 1$. Substituting in this equation the values of M and T we have $20.4\ K = 1$, whence $K = 0.049$.

At the start of the reaction the value of M is 0; this gradually increases to 1 and remains constant. During this period of increase the value of M may be calculated as follows: When M has reached its constant value ($M = 1$) let us suppose that the reaction $S \rightarrow M$ suddenly stops while $M \rightarrow P$ continues; we shall find that if T minutes have elapsed after this occurrence, the amount of M which has disappeared is $1 - e^{-KT}$ (see Fig. 5). If the reaction $S \rightarrow M$ had not

stopped it would have produced enough of M so that (in spite of the fact that M is constantly decomposing) the amount of M remaining at the time, T, would be just enough to balance the loss, or $1 - e^{-KT}$. Hence if we start with nothing but S (the values of M and of P being 0) the amount of M present after the lapse of any given time T will be $1 - e^{-KT}$ and the amount of P will be

$$P = KT - (1 - e^{-KT}).$$

This is the same as the equation

$$\frac{P}{A} = T - \frac{1}{K} + \frac{1}{K}e^{-KT},$$

when in the latter we put $K = A$ as was done in making the calculations given in Table I. Hence when we substitute the value $K = 0.049$ in the equation $P = KT - (1 - e^{-KT})$, we obtain the values already given in Table I.

If the chlorophyll takes part in the reaction by decomposing or by combining (as some recent evidence indicates), we might suppose that S represents inactive chlorophyll, M active chlorophyll, and P a derived substance which combines with CO_2. At present it does not seem profitable to attempt a more extended discussion of this question. But it may be pointed out that (as one of us has recently emphasized)[21] consecutive reactions of the type here discussed are to be looked upon as the rule, rather than as the exception, in living matter.

It is evident that either of the theories developed above gives a quantitative explanation of the results. Both seem to be based on reasonable assumptions. Future investigation must decide which is more useful.

In any event, it is clear that much is to be learned concerning the dynamics of photosynthesis, and it is hoped that the considerations here set forth may be of value in this connection.

SUMMARY.

Minute amounts of photosynthesis in marine plants can be accurately measured by adding a little phenolphthalein to the sea water,

[21] Osterhout, *J. Biol. Chem.*, 1917, xxxi, 585; xxxii, 23.

and observing changes in the color of the indicator. In the case of fresh water aquatics bicarbonates are added.

By this method it is found that *Ulva* which has been kept in the dark begins photosynthesis as soon as it is exposed to sunlight and that the rate steadily increases until a constant speed is attained.

This may be explained by assuming that sunlight decomposes a substance whose products either catalyze photosynthesis or enter directly into the reaction.

Quantitative theories are developed in order to account for the facts.

A METHOD OF STUDYING RESPIRATION.

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, July 2, 1918.)

In 1915 the writer suggested to Dr. Haas the desirability of experiments with indicators with a view to measuring the amount of CO_2 given off by organisms. The method finally developed by Dr. Haas[1] in the writer's laboratory consists in adding the indicator directly to the liquid in which the organism is placed. The indicator is usually present at the start, but in some instances it is not added until after the CO_2 has been produced.

The method is simple, accurate, and extremely satisfactory but it has obvious limitations. It is restricted to the use of aquatic organisms and furthermore it does not permit us to study the effect upon respiration of reagents which have a pronounced acid or alkaline reaction. There are also difficulties in using organisms or tissues which give off alkali or acid (other than CO_2). In addition, the use of small suspended organisms which color the solution must be avoided. A further disadvantage is that some reagents cause organisms to give off coloring matters which interfere with the results. The use of toxic indicators also presents difficulties.

These difficulties may be obviated by means of an apparatus designed by the writer. The purpose of the present article is to make clear the principle employed and to describe a simple form of the apparatus without mentioning other forms or discussing the modifications of mechanical details which are of importance for special purposes.

The apparatus shown in Fig. 1 consists of a bottle, A, in which the organisms are placed; the CO_2 which they exhale passes out of A through D into the indicator solution[2] in the Pyrex glass tube, B,[3]

[1] Haas, A. R. C., *Science*, 1916, xliv, 105.

[2] The tube which dips into the indicator solution should be of Pyrex glass. This glass is chosen because the amount of alkali given off is negligible.

[3] This tube should be about ¾ inch in diameter; the stopper should be covered with a paraffin which does not give off acid.

returning through the rubber syringe, F,[4] into A. A constant circulation[5] is kept up by the motion of K, each downward movement of which compresses the syringe, F, forcing a stream of bubbles[6] through the indicator solution in B; K is attached at one end to the hinge, L, and at the other to the connecting rod, G, which moves when H is made to revolve by means of a small motor. The syringe,

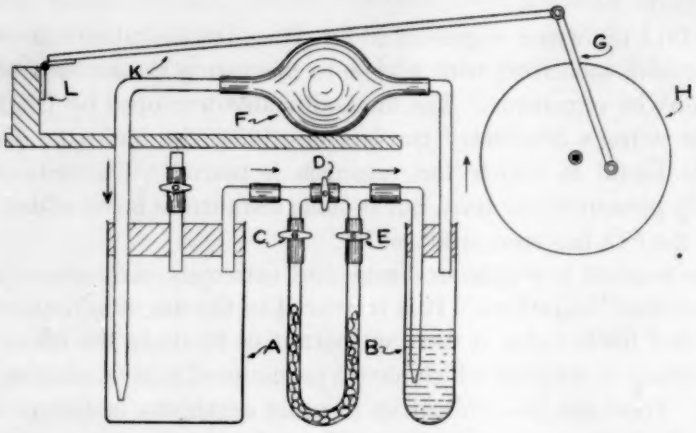

FIG. 1. Apparatus for the measurement of respiration and of photosynthesis. The organisms are placed in A; by compressing the syringe, F, the CO_2 is forced through the indicator solution in B, returning through F into A. The wheel, H, is caused to rotate by means of a motor; the resultant motion of K compresses F and keeps up a constant circulation of gas.

[4] This has valves at the outlet and inlet. In place of the syringe a tambour-bellows, pump, miniature fan, or other simple device may be employed.

[5] It is desirable to have the gas circulate at a constant rate. The rate may be controlled in a number of ways; it may be tested by observing the rate at which the color of the indicator changes, when a stream of air, free from CO_2, runs through it.

[6] If the stream of bubbles does not flow satisfactorily some of the solution may be removed so that the top of the tube is brought nearer to the surface. Inclining the tube, B, may assist in freeing the bubble as it issues from the tip of the tube. The compression of the syringe may also be increased and a syringe with stiffer walls may be used. If this does not produce the desired result the elasticity of the system must be increased by making the rubber connections longer, or by using softer rubber; or, better, a small elastic bulb may be introduced at any convenient place.

F, rests upon a board which is held in position by a support not shown in the drawing. The amount of compression of F can be varied either by shifting F or by altering the point at which G is attached to H.

A and B are provided with tightly fitting stoppers into which the tubes are fastened in air-tight fashion. All other connections should likewise be air-tight.

The carbon dioxide produced by the organism is absorbed with surprising rapidity by the indicator solution, provided a rapid circulation of air is maintained. This is evident from the fact that when known amounts of CO_2 are introduced into A the CO_2 is at once carried over into B and equilibrium is quickly established.

Ordinarily it is not important to know the absolute amount of CO_2 produced by the organism, since we are concerned only with comparative values. If for example we wish to compare the normal rate of respiration with the rate under the influence of ether, we proceed as follows: We place the organisms in A and close the clamp, D, opening C and E so that the air passes through the U-tube[7] (containing an absorbent of CO_2, such as lumps of NaOH)[8] into the indicator solution in B. The indicator solution may consist of distilled water (free from CO_2) to which enough NaOH has been added to make its pH value about 7.3; phenolsulfonephthalein is added, so that the solution becomes pale pinkish in color. As the gas passing into B is practically free from CO_2 little or no change will be produced by its circulation; it should, however, be allowed to circulate until the color of the indicator has become constant.[9] The clamps, C and E, are now closed and D is opened, allowing the CO_2 given off by the organisms to pass into B. The time required to produce a clearly marked change in the color of the indicator is noted and buffer solutions (contained in Pyrex glass tubes of the same size as B and having the same concentration of indicator) are selected which match the color of the indicator at the start and finish. The clamp D is now closed; C and E are opened, thus washing the CO_2 out of B until the indicator returns to

[7] In place of the U-tube a tower or potash bulb may be employed.

[8] When a volatile reagent, as ether, is added, the substance in the U-tube must be one which does not absorb the reagent.

[9] Unless the joints are tight CO_2 may leak in.

the original color. This is repeated until the normal time of respiration is established (unless it is fairly constant the experiment should be rejected).

The reagent is now introduced into A (by means of the short tube) and the time is ascertained which is required to produce the same change in the color of the indicator. A comparison of this with the normal time gives the relative rate of respiration under the influence of the reagent.

The amount of CO_2 produced by the organisms is ascertained by comparing[10] the color of the indicator with the colors of a series of buffer solutions[11] having the same concentration of indicator and contained in Pyrex glass tubes of the same size as B. This determines the pH value of the solution. In order to find out how much CO_2 must be evolved to produce a given change in the color of the indicator we introduce known amounts of CO_2 into the apparatus[12] by means of a device which has recently been described.[13] The gas is made to circulate in the apparatus until the introduced CO_2 is thoroughly mixed with the air and with the indicator solution, so that equilibrium is established between the latter and the CO_2 in the apparatus. We know that this has occurred when continued circulation fails to produce any further change in the color of the indicator.

Instead of introducing gaseous CO_2 we may introduce a solution whose CO_2-content is known from its pH value.

The change in the color of the indicator produced by a given amount of CO_2 will vary according to the volume of air in the apparatus (the amount of indicator solution being constant). When determinations have been made for a number of volumes, intermediate values may be obtained by interpolation. As a rule there will be no changes in volume except those produced by variations in the volume of the organisms which are introduced into the apparatus.

Ordinarily the respiration of aquatic organisms may be studied by the method described by Haas[1] but such organisms as impart a color to the solution or give off acid (other than CO_2) or alkali may be

[10] This should be done, if possible, under a "Daylight" lamp.

[11] Cf. Osterhout, W. J. V., and Haas, A. R. C., *Science*, 1918, xlvii, 420.

[12] This can be done by means of the short tube in the stopper of A.

[13] Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxv, 237.

studied by means of the apparatus here described. In this case A is partly filled[14] with liquid,[15] and due allowance must be made for this in calibrating the apparatus with respect to absolute amounts of CO_2 required to produce a given change of pH value. This calibration must be revised if any reagent is added which has a pronounced buffer action; this should also be done when A is filled with gas if any volatile substance with a pronounced buffer effect (or with an acid or alkaline reaction) is used.

In general it would seem to be desirable to have A large enough to contain a number of organisms, so as to reduce as much as possible the irregularities which may occur when small numbers are used. But if the amount of CO_2 given off is small, it may be necessary to reduce the size of A by substituting a U-tube for the bottle. The size of F may also be reduced by substituting a smaller bulb, or by using in place of a bulb a tambour provided with valves.

Whenever it becomes desirable to sweep out the exhaled CO_2 and to fill the apparatus with ordinary air, this can be easily and quickly accomplished, without disturbing the organism. It is only necessary to remove the U-tube and to close the screw clamp, D, (which is usually open) and open the screw clamps, C and E, (which are ordinarily closed) and then to start the motor and allow the gas to circulate, passing out of the apparatus at E. Air will then enter at C and will quickly wash out the excess of CO_2. When this is accomplished (as shown by the color of the indicator), C and E are closed and D is opened.

Ordinarily it will be found desirable to remove the excess of CO_2 at frequent intervals. In this case phenolsulfonephthalein will be found useful as an indicator.[1] If, however, CO_2 is allowed to accumulate, other indicators or mixtures of two or more indicators may be used to measure the lower pH values.

[14] Except in cases where the organisms respire normally when merely moistened and kept in a saturated atmosphere. Ordinarily the air will become partly saturated with the moisture taken up from the indicator solution. If this is not desirable, in the case of non-aquatic organisms, it can be obviated by adding something to the indicator solution to lower vapor tension or by drying the gas on its way from B to A.

[15] The tube by which the gas enters A is drawn to a point and brought down to the bottom of A to permit the gas to bubble through any liquid contained in A.

In case it is desired to withdraw a sample of gas in order to determine the amount of O_2 consumed, it is only necessary to intercalate a glass tube in the neighborhood of D, with stop-cocks at each end which can be closed when the tube is removed.

It is evident that this method is just as applicable to photosynthesis[4] as to respiration. In studying photosynthesis it may be desirable to introduce known amounts of CO_2 into the apparatus at the beginning of the experiment.

In conclusion it may be added that in carrying out such investigations it is desirable to compare the times required to produce equal amounts of CO_2 rather than to compare the amounts of CO_2 produced in equal times. The former method compares reaction velocities while the latter may not.

SUMMARY.

An apparatus is described which makes it possible to measure rapidly and accurately small amounts of CO_2 given off by organisms of all kinds. The apparatus can also be used to measure photosynthesis.

THE ANTAGONISM BETWEEN THYMUS AND PARATHYROID GLANDS.

By EDUARD UHLENHUTH.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 16, 1918.)

INTRODUCTION.

It is well known that the extirpation of the parathyroids in mammals is followed by tetanic convulsions. This led to the conclusion that certain tetany-producing toxins are formed in the body, which in normal animals must be either removed or turned into non-toxic substances by the parathyroid glands. The origin of these toxic substances, however, is unknown. Though tetanic convulsions and other symptoms of tetany have been reported repeatedly to occur after injection of thymus extracts, in the frog as well as in mammals,[1] no attention has been paid to this fact as possibly containing the solution of the problem in which organ the tetany-producing substances might be manufactured. The experiments presented in this article seem to indicate that they are contained in and produced by the thymus gland. This harmonizes with the well known fact that tetany is a disease of infants.

In the spring of 1916, about thirty salamander larvæ of the species *Ambystoma maculatum*[2] were fed exclusively on calf's thymus. Each single larva after some time began to suffer from severe tetanic attacks. Since the larvæ of salamanders do not possess parathyroids this observation seemed to be of considerable interest, and, in the fall of 1916, calf's thymus was fed to larvæ of another species of sala-

[1] See Biedl, i, 279, 301 ff.

[2] In the terminology to be employed in the classification of amphibians the nomenclature as worked out by Stejneger and Barbour in their new check list was applied here. According to this the old species *Amblystoma punctatum* corresponds to the new species *Ambystoma maculatum*.

manders, *Ambystoma opacum;* again each single individual after a certain time came down with tetanic attacks. In 1917, the experiments were repeated with the same result on both species. It is, therefore, evident that the thymus gland contains a substance which produces tetanic convulsions in the larvæ of *Ambystoma maculatum* and *opacum*.

Tetanic Symptoms.

The tetanic symptoms exhibited by salamander larvæ fed with thymus closely resemble the symptoms produced by parathyroidectomy in mammals.[3] The first attacks in the larvæ are confined to the hind portions of the body. In general, this part of the body suffers more than any other during the entire tetanic period, and when the acute attacks become less and less frequent and severe it is again the hind portions of the body which alone are attacked. Within several days after the onset of acute tetany the entire muscular system exhibits severe clonic convulsions. Each single attack begins at the tail and spreads toward the head; the tremors are severest in the legs and in the muscles of the lower jaw. During each attack the mouth is thrown widely open. When the attack begins the animals are thrown on one side. Besides the clonic convulsions a tonic spasm of the entire body is observed; the legs are stretched out and the body is bent with its concavity towards the back. During the attack the animals discharge much air and the vessels of the skin become very red.

In the early stages of the disease the convulsions are induced only upon stimulation which is best effected by removing the larvæ from the water, placing them on filter paper, and pinching the legs or the tail slightly. Each attack lasts only a short time, the larvæ recovering after from 2 to 3 minutes. Later any attempt of the larvæ to swim or to snap at a piece of food suffices to induce an attack and some individuals may float for several days on one side, being rigid from tonic spasm.

8 to 14 days after the first attack the hind legs begin to show signs of a permanent tonic spasm; the legs are stretched backward and become twisted around their longitudinal axis with the inner surface pointing upward; the feet follow this movement and finally the fore legs undergo a similar change.

[3] See Biedl, i, 79 ff.

Relation between Tetany and Development.

Tetany cannot be produced by thymus feeding before a certain developmental stage is reached by the larva; namely, that at which probably the secretive power of the animal's own thymus glands is established. This stage of development corresponds to that of the full development of the toes of the larvæ.

Six larvæ of *Ambystoma opacum* (*opacum*, 1917, B), from North Carolina, hatched in the laboratory from eggs of the same female, were kept at approximately 25°C. and fed on calf's thymus from the 19th day after hatching. The animals were tested for tetany regularly on a definite day every week, for the purpose of recording the percentage of animals suffering from tetany; clonic convulsions of the entire body as well as of the hind portion only were considered as indication of tetany. No animal suffered from tetany when examined the first time after the commencement of the thymus feeding. With the progress of development (as indicated by the development of the toes) the percentage of animals suffering from tetanic convulsions increased and reached a first maximum at the time when all toes were developed. No animal had convulsions until all the toes of the fore legs and at least the third toe of the hind limbs were developed.

In a second series of experiments, with eight *Ambystoma opacum* larvæ (*opacum*, 1916, B) hatched from eggs of the same female and kept at approximately 25°C., thymus feeding was started on the 14th day after hatching. None of the larvæ of this series showed any signs of tetany before the full number of toes had developed. In a third series (*Ambystoma opacum*, 1916, D) consisting of eight larvæ from eggs of the same female and of the same age as the larvæ of the previous series, this becomes still more evident. Though the larvæ of Series D, 1916, were fed on thymus simultaneously with Series B, 1916, convulsions developed 4 weeks later than in Series B, 1916. Development was retarded by keeping the larvæ in low temperature (15°C. approximately).

In *Ambystoma maculatum* similar results were obtained. In one instance (*Ambystoma punctatum*, Series K, 1917) six larvæ, all hatched from eggs of the same female and kept at approximately 25°C., were fed on thymus exclusively from the 13th day after hatching. The first acute attacks were observed 5 weeks after hatching.

Similarly it was found in *Ambystoma opacum* as well as in *Ambystoma maculatum* that the acute tetanic attacks disappear entirely after a certain stage is reached, though the animals continued to be fed exclusively on thymus gland. When the larvæ reach a stage in which they resemble normal larvæ ready for metamorphosis, it becomes more and more difficult to induce an acute tetanic attack and no animal ever showed such attacks after it had metamorphosed, in spite of thymus feeding.

Thymus Gland Contains a Tetany-Producing Substance.

The question arises: Why does the thymus gland when fed to salamander larvæ cause tetany only during a certain developmental period, and why is it ineffective before and after this period? In order to give an answer to this question it was necessary to make a histological examination of the larvæ.

As regards the beginning of tetany several larvæ which had been fed thymus were examined histologically just before and after the beginning of tetany. In a number of *Ambystoma opacum* larvæ of the same age and hatched from eggs of the same female (*Ambystoma opacum*, 1917) tetany was observed in a few individuals as early as 4 weeks after hatching; in these few individuals, however, it did not reappear until 6 to 7 weeks after hatching, and at this time the majority of the larvæ developed tetany. At this time larvæ of this set with and without tetany were killed and preserved for examination to find in which point they differed. A preliminary microscopical study of the anatomy and histology of one tetanic and two pretetanic larvæ demonstrated a noteworthy difference in regard to the developmental state of the thymus glands, but in no other respect.

It was found that the larvæ which had not yet developed tetany had smaller thymus glands than the larva which had already fallen a victim to tetany. The thymus glands of the latter larva are more than twice as large as those of the pretetanic larvæ, though the difference in length of the entire animals is only 33 per cent, the tetanic specimens being the larger ones. This is shown in Fig. 1 in which the three left thymus glands of a pretetanic larva (left vertical row)

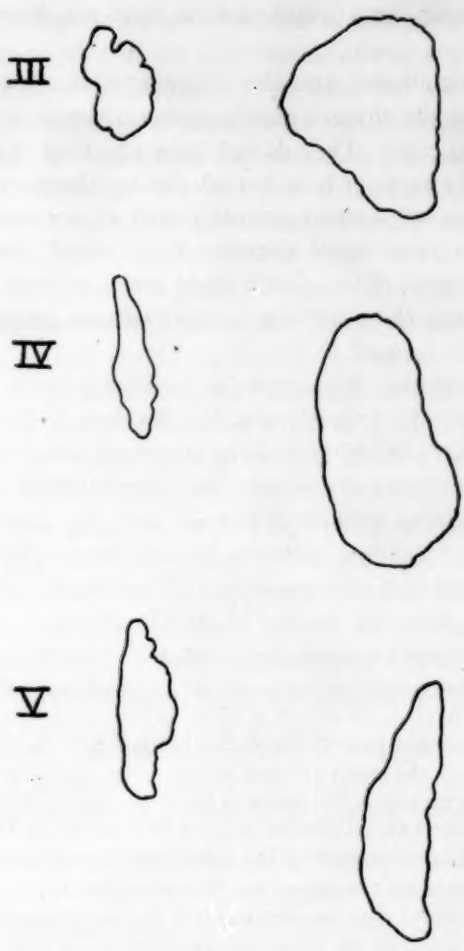

FIG. 1. Comparison between size of the primordial thymus of a pretetanic larva of *Ambystoma opacum* (left vertical row) and a larva of the same species shortly after the beginning of acute tetanic convulsions (right vertical row). It will be seen at a glance that the thymus glands of the latter animal are much larger than those of the non-tetanic larva. The drawings were made with a camera lucida at 490 linear magnification from the largest cross-section through each of the glands.

and of the tetanic larva (right vertical row) are drawn at the same magnification.[4]

Still more significant are the differences in structure. In the pretetanic larvæ the thymus glands represent small accumulations of purely epithelial cells. They do not show any kind of differentiation. Their epithelial character is indicated also by the presence of considerable quantities of pigment granules such as are found in the epithelium of the pharyngeal pouches from which the glands have separated. In most of the glands there are no mitoses to be found at all, while in some there are one to two mitoses present. No membrana propria is formed.

Compared with the thymus of the pretetanic larvæ, in which none of the characters of a true thymus has developed, five of the glands of the tetanic larva show some of the aspects of a real thymus in as far as they contain elements which are characteristic of that organ. Again no membrana propria is formed, but the glands consist of a variety of cells. Leaving aside the question as to whether or not the newly developed cell elements are of mesenchymal origin,[5] it is sufficient to notice that several kinds of cells now constitute these glands; two of them are recognized clearly as the main elements making up the fully developed thymus of an *Ambystoma opacum* larva.

[4] The first and second pairs of the thymus glands have disappeared already in this stage from both the pretetanic and tetanic larvæ, and only the third, fourth, and fifth pairs are present. According to Maximow and to Baldwin the first and second pairs are found absent already in larvæ of from 11.5 to 13.0 mm. in length.

[5] Concerning the development of the amphibian thymus glands and the origin of their cell elements see the papers by Maximow, by Dustin, and by Baldwin. It should be mentioned that the development of the thymus glands in *Ambystoma opacum* was found by the writer to correspond in the main with the description as given by Maximow and Baldwin for *Ambystoma tigrinum* and *Ambystoma maculatum* respectively. The size of the larvæ, however, corresponding to the various developmental stages of the glands was found to be much more variable and in general larger. In a normal stock animal of *Ambystoma opacum*, for instance, the right gland of the third pair was found to be still connected by an epithelial stalk with the brancho-pharyngeal epithelium and of purely epithelial character, though the larva has a length of over 37 mm., while Maximow and Baldwin found the glands fully separated in larvæ of from 10 to 12 mm.; Maximow saw the commencement of mesenchymal immigration in larvæ of about 10 mm. in length, and Baldwin in larvæ of 19 to 20 mm in length.

Correspondingly the epithelial character of the glands is lost; this finds its expression also in the fact that pigment granules have disappeared altogether. In addition a considerable number of mitoses may be found, which mostly seem to be cell divisions of the newly arrived elements. Of particular importance is the fact that in one of the glands of the fifth pair, which I found to be frequently ahead of the third and fourth pairs in development, the cell plasma of a number of cells stains pink, and here one nucleus which also is stained pinkish exhibits a swollen appearance, characters which are ascribed by Maximow to the formation of the medulla of the thymus.

The thymus glands of the pretetanic larvæ are therefore mere accumulations of epithelial cells and do not possess any structures to indicate that they would be capable of exerting any of the functions of a thymus, while in the tetanic larva at least some of the glands resemble a true thymus and probably have commenced to behave as such with regard to function. From these observations we may conclude that the thymus feeding does not begin to call forth tetany in salamander larvæ until the thymus glands of the larvæ themselves are able to secrete. This suggests that tetany is the effect of the combined action of the secretion of certain substances by the thymus glands of the larvæ and of substances introduced into the larva by feeding it with the thymus gland from other animals.

Prevention of Tetany by the Parathyroids.

It is well known that salamander larvæ do not possess parathyroids; these glands develop during metamorphosis. Though the end of the tetanic period coincides approximately with the development of the parathyroid glands, yet larvæ which had ceased to show convulsions for some time apparently possessed no parathyroids when examined histologically. It is, however, possible that in spite of this in such larvæ certain processes had commenced which would soon have led to the formation of these glands and that these processes were already able to check tetany.

A connection between the development of the parathyroids and the cessation of tetany seems probable on account of the fact that tetany

was never observed after metamorphosis and after the development of the parathyroids. This suggestion finds support in the fact that in the larvæ of frogs and toads, which develop their parathyroids soon after hatching, thymus feeding never produces tetanic convulsions.

A Second Mechanism to Counteract the Action of the Thymus in Salamander Larvæ.

If the parathyroids are responsible for preventing tetany in *Ambystoma opacum* and *Ambystoma maculatum*, the question may be asked why the larvæ of these salamanders are not normally victims of tetany, since they possess six well developed thymus glands and no parathyroids. It is necessary to assume that the larvæ must possess some other mechanism capable of inhibiting the action of their own thymus glands but inadequate when an excess of thymus substance is introduced into the larva.

That such a mechanism is present becomes evident from the behavior of the larvæ of a third salamander species (*Ambystoma tigrinum*) closely related to the two others. It is surprising that the larvæ of *Ambystoma tigrinum* never develop tetany even if they are fed exclusively on thymus gland of which they eat enormous quantities. *Ambystoma tigrinum* larvæ were fed from about the 14th day after hatching, on calf's thymus exclusively; some of them were kept at 25°C., others at 15°C. One of these larvæ has not metamorphosed yet, 60 weeks after hatching; it has been fed on thymus for more than a year. None of these larvæ ever showed any signs of tetany.

Yet the larvæ of the species *Ambystoma tigrinum*, like those of the two other species, do not possess parathyroids. Evidently they must have some other mechanism to counteract the tetanic effect of the thymus; and this mechanism must be sufficient not only to prevent tetany from the animal's own thymus but also tetany from an excess amount of thymus when introduced with its food.

CONCLUSIONS.

From the facts stated in this paper it is evident that the thymus gland of mammals contains a substance which is capable of producing

tetany when fed to the larvæ of certain species of salamanders (*Ambystoma opacum* and *Ambystoma maculatum*). As long as the larvæ have not developed their own thymus glands, they are able, by means of some mechanism, to counterbalance the tetanic action of the thymus substance introduced in their food. When, however, the secretion from their own thymus glands is added to the thymus material introduced with the food, this mechanism of preventing tetany becomes inadequate and tetany ensues. In the larva of a third species of salamander, *Ambystoma tigrinum*, this mechanism will prevent tetany even when the larvæ are fed on thymus.

In mammals the parathyroids are known to prevent tetany and are supposed either to absorb the tetany-producing substance and thus prevent its action or to change it into another non-toxic substance. It is at least probable that in the amphibians the parathyroids play the same rôle. Larvæ of anuran amphibians, which develop their parathyroids soon after hatching, never show tetanic convulsions if they are fed on thymus, but in certain species of salamanders, whose parathyroids develop only during metamorphosis, the larvæ invariably have tetanic convulsions upon thymus feeding, while the metamorphosed animals never show tetany.

But in addition to the parathyroids the salamanders must possess still another mechanism which during the larval period inhibits the production of tetany by the animal's own thymus glands. In the larvæ of *Ambystoma opacum* and *Ambystoma maculatum* this mechanism is sufficient only to prevent tetany from the animal's own thymus, while in the larvæ of *Ambystoma tigrinum* it is capable of preventing tetany even when the larvæ are fed with thymus.

If the thymus is the organ by whose action tetany is produced, we can understand why tetany in human beings occurs far more frequently in children than in adults, since in the latter the thymus gland is replaced, at least to a great extent, by connective tissue. The relation of thymus to tetany may also possibly explain the occurrence of tetany during pregnancy; while the parathyroids of the mother may be sufficient to prevent tetany from her largely atrophied thymus, they may not be sufficient to prevent tetany from the excess of thymus substance furnished by the fetus to the blood of the mother.

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FURTHER PROOF OF THE EXISTENCE OF A SPECIFIC TETANY-PRODUCING SUBSTANCE IN THE THYMUS GLAND.

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(Received for publication, July 8, 1918.)

In the preceding note[1] it has been shown that salamander larvæ which are fed exclusively on calf's thymus gland develop tetanic convulsions. Several facts were mentioned supporting the idea that this phenomenon is identical with the tetany observed in mammals after parathyroidectomy; it was also made probable by our experiments that the tetanic convulsions, produced in the salamander larvæ, were due to the action of a specific substance contained in the thymus, inasmuch as the thymus feeding did not become effective until the primordial thymus glands of the larvæ had reached a stage in which they assume some of the structures of true thymus glands.

Instead of assuming that the thymus gland secretes a toxin causing tetany, it might be argued that the effects of thymus feeding were due to the absence from the thymus of some substances which are necessary to make up a complete diet for the salamander larvæ, for in the experiments reported in our previous paper the larvæ were fed exclusively on thymus. In other words, it might be argued that the tetany produced in our experiments was in reality a deficiency phenomenon.

While formerly it was generally assumed that the thymus belongs to the glands with internal secretion, doubts have recently arisen in this regard. After it had apparently been demonstrated by some authors that the thymus contains substances which stimulate the growth of tadpoles of frogs and toads[2] in a specific way, Swingle[3] has

[1] Uhlenhuth, E., *J. Gen. Physiol.*, 1918, i, 23.

[2] Gudernatsch, J. F., *Arch. Entwcklngsmechn. Organ.*, 1913, xxxv, 457; *Am. J. Anat.*, 1913–14, xv, 431.

[3] Swingle, W. W., *J. Exp. Zool.*, 1917–18, xxiv, 521.

recently published feeding experiments performed on tadpoles of frogs which seem to prove that the feeding of such larvæ with thymus gland of mammals has no influence whatever on growth. The writer's[4] own experiments on salamanders demonstrate clearly that during the larval period of these animals, thymus when given as food may considerably change the rate of growth, but that this is not due to a specific influence of that gland upon growth.

It is, therefore, of interest that we are able to show that the tetany produced in thymus-fed salamander larvæ is not due to a deficiency in the thymus of one or several substances, but is due to the presence in the thymus of a specific tetany-producing substance.

EXPERIMENTAL.

If tetany in salamander larvæ fed on thymus is due to the absence from the thymus of one or more substances, it is clear that the addition of a sufficient amount of normal food to the thymus diet should prevent the development of tetanic convulsions. It was found, however, that this is not the case.

Thirty larvæ of the species *Ambystoma maculatum*—from the same mother and of the same age—were fed on thymus from about the 5th week after hatching. The larvæ developed tetany very soon; convulsions were noticed as early as 15 days after the first thymus meal. At the end of the 3rd week, the series was divided into two lots, each containing fifteen individuals. One group was fed on thymus exclusively, the other one was fed alternately one day on thymus and one day on earthworms, the latter constituting a complete diet in the case of the salamanders.

The result of this experiment was that tetany is not stopped by the addition of a sufficient amount of complete diet, but on the contrary developed still further towards a maximum. Therefore, the tetanic convulsions resulting from thymus feeding are not due to a deficiency in the diet but must be due to a specific tetany-producing substance contained in the thymus gland.

It was also noticed that in the mixed food series the tetany period was shorter than in the thymus series and that the percentage of ani-

[4] Uhlenhuth, *Proc. Soc. Exp. Biol. and Med.*, 1917, xv, 37.

mals suffering from tetany was smaller on a mixed diet than on pure thymus diet. This is due to several factors.

First, it was pointed out in a previous article[1] that the salamander larvæ, though they have no parathyroids, must possess some other mechanism capable of doing away with a certain limited amount of the tetany-producing poison; otherwise even normal larvæ not fed on thymus should show tetany as soon as their own thymus glands are sufficiently developed. In one species (*Ambystoma tigrinum*) this mechanism is strong enough to prevent tetany even if by means of feeding thymus exclusively the body is flooded with a large amount of the tetany toxin. Hence, it is not surprising that if a smaller dose of the toxin is introduced into the body, the larvæ should be capable of removing—or antagonizing—so much of the toxin that the remaining part may be less effective than a maximum dose would be. Hence the fact that tetanic attacks occurred less frequently in each single individual on a mixed diet than on an exclusive thymus diet is due to the difference in the amount of thymus substance.

A second factor is still more important as regards the decrease of tetany in the mixed food series than the first factor mentioned. It has been pointed out in another communication[1] that larvæ of the species of *Ambystoma maculatum* and *Ambystoma opacum* cease to show tetanic convulsions after metamorphosis, probably because of the action of the parathyroids developing during metamorphosis. Metamorphosis started in the mixed food series 7 weeks earlier than in the pure thymus series, and was finished in the former series 19 weeks earlier than in the latter series.

We have alluded to the fact that some authors believe they have demonstrated that thymus feeding retarded the metamorphosis of the tadpoles of frogs and that this effect was specific. This conclusion is not correct. It became clear in the course of our study of amphibian metamorphosis[5] that the retardation of metamorphosis upon exclusive thymus feeding is probably not due to a specific effect of the thymus, but to a deficiency in the diet. If retardation of metamorphosis upon thymus feeding were due only to the absence of certain substances from the thymus and tetany were due to the presence of certain toxic

[5] Uhlenhuth, E., *J. Exp. Zool.*, 1918, xxv, 135.

substances in the thymus, we should expect that upon addition of a complete diet to the thymus food tetany would still occur, but metamorphosis would take place in the normal way and at the normal time. In fact, metamorphosis started even a few days earlier than in a control series consisting of thirty larvæ of the same age. Thus by adding normal food to the thymus diet the specific actions of the thymus may be sifted out from the non-specific ones.

The tetany produced by exclusive thymus feeding is a specific effect of the thymus due to the existence of a tetany-producing poison in that gland, while the retardation of metamorphosis in the case of an exclusive diet of thymus is due to a deficiency in the diet.

CONCLUSIONS AND SUMMARY.

The effect of the thymus gland in producing tetany is due to a specific tetany toxin produced by and contained in the thymus, and the thymus gland must be added to the group of glands for which the function of internal secretion has been demonstrated.

DIFFERENCE IN THE ACTION OF RADIUM ON GREEN PLANTS IN THE PRESENCE AND ABSENCE OF LIGHT.

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(Received for publication, July 18, 1918.)

Experiments by Willcock[1] on the effect of radium radiations on *Hydra viridis* and *Hydra fusca* show that the green form is far more resistant to rays than the brown. The green hydras are not killed by an exposure of 4½ hours to 50 mg. of radium bromide; the brown hydras, on the other hand, die after "comparatively short exposures." I have repeated this experiment several times and have obtained the same results. Willcock also found that protozoa containing chlorophyll are uniformly more resistant than those which have none. Since these experiments were carried on in daylight, it seemed possible that the difference in the reaction between the two classes of animals experimented upon might be due to the activity of the chlorophyll.

If this assumption is correct, we should expect that plant cells radiated in complete darkness, when the chlorophyll is not active, would be more sensitive to the rays than in the light. To test this point I have made a number of experiments on *Spirogyra* and *Volvox*.

The radium (20.4 mg. of element, enclosed in a thin-walled silver tube) was placed in a watch-glass filled with water, and the *Spirogyra* filaments were laid across it. In the light, the cells nearest the tube began to show a typical phenomenon of disintegration in 2 to 3 hours. This disintegration consists in the following.

The chlorophyll bands first contract somewhat, losing their spiral arrangement. A few minutes later they resolve into an irregular heap of chlorophyll in one end of the cell. In the light, this phenomenon began to be noticed after an exposure of from 2½ to 3 hours. The

[1] Willcock, E. G., *J. Physiol.*, 1904, xxx, 449.

disintegration became visible in many cells simultaneously, and within 5 minutes after the disintegration began to be visible in a few cells, practically all the cells in the vicinity of the radium were thus affected.

In the dark, this phenomenon of disintegration commenced in about one-fourth of the time; namely, after an exposure of from 35 to 70 minutes, the average being 45 minutes.

Colonies of *Volvox* were exposed in a small glass tube in which the radium preparation was placed. In the light, the colonies became motionless in 2 hours; in the dark, the same condition was attained in 55 minutes. These periods represent the average of many trials.

It is very evident that the life of the cell is prolonged by some condition connected with photosynthesis; a more definite statemen cannot be made on the basis of these preliminary experiments.

AMPHOTERIC COLLOIDS.

I. Chemical Influence of the Hydrogen Ion Concentration.

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(Received for publication, July 26, 1918.)

I.

It is often stated (Pauli, Höber, and others) that both ions of a neutral salt affect the physical properties of an amphoteric electrolyte (ampholyte) simultaneously and in the opposite sense; so that the total result is the algebraic sum of the opposite action of the oppositely charged ions of the neutral salt.[1] The writer has been able to show by experiments on gelatin and on pig's bladder that this statement is not correct, but that apparently only one of the two ions of the neutral salt acts upon the ampholyte; namely, the one which has the opposite electric charge from that of the ampholyte.[2]

The hydrogen ion concentration of the gelatin solution used by the writer was 10^{-7} (or in Sörensen's logarithmic symbol pH = 7.0) and this gelatin reacted with neutral salts as if it were an anion capable of combining with the cation of a neutral salt. When, however, powdered gelatin is treated for some time with an acid, *e.g.* HCl, gelatin chloride (or hydrochloride) is formed, which is supposed to dissociate electrolytically into a positive gelatin ion and a negative chlorion. The writer found that when the supernatant

[1] This and other erroneous statements current in colloid chemistry are due to the fact that the previous investigators always studied the action of neutral salts upon proteins in the presence of the salt. The writer removed these salts after they had a chance to act on the gelatin. This procedure is necessary on account of the fact that the manifestation of the effect of the electrolyte upon the gelatin is repressed in the presence of the electrolyte.

[2] Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 343; 1918, xxxiii, 531; xxxiv, 77, 395, 489; 1918, xxxv, 497.

acid is washed away from such powdered gelatin it is affected only by the anion of a neutral salt, while the cation of the salt used has apparently no effect.

The writer found that when powdered gelatin is treated for some time with a base, *e.g.* NaOH, the gelatin (after the supernatant NaOH is washed away) is only capable of combining with the cations of neutral salts, while the anions of neutral salts are without effect.

It seemed necessary to find out where the turning point for these two different modes of reaction lies, whether at the point of neutrality of the solution or at the isoelectric point of gelatin. The fact that gelatin solutions at the point of neutrality (pH = 7.0) react with neutral salts as if they had previously received an alkaline treatment, suggests that the turning point lies at a more acid reaction of the gelatin and since the isoelectric point lies at a more acid reaction it was *a priori* likely that the isoelectric point for gelatin might be the turning point.

Amphoteric electrolytes are defined by the fact that they can dissociate electrolytically as acids as well as bases. When they dissociate as acids they are supposed to send more H ions than OH ions into solution and the ampholyte ion migrates in the electric field to the anode. When they dissociate as bases they are supposed to send more OH ions than H ions into solution, and the ampholyte migrates in the electric field as a cation. At the isoelectric point the ampholyte is supposed to send out an equal number of H and OH ions and it migrates in neither direction. Gelatin is naturally a stronger acid than base and hence migrates to the anode. By increasing the hydrogen ion concentration of the surrounding solution through the addition of some acid the dissociation of gelatin as acid can be repressed to the level of its dissociation as a base. This is the isoelectric point at which it migrates in neither direction and at which the total number of ions it sends into a solution is a minimum. With the addition of more acid it migrates to the cathode. Michaelis determined the isoelectric point of gelatin solutions by the migration method and found it in the neighborhood of $C_H = 2.10^{-5}$ (or in Sörensen's logarithmic symbol pH = 4.7).[3] Since the solution of gelatin used had a pH of 7.0 it was on the alkali side of the isoelectric point. We shall

[3] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

show in this paper that gelatin on the alkali or less acid side of its isoelectric point can combine only with the cations of neutral salts, as we had actually found empirically in our previously published papers.

Moreover, it will follow from the experiments to be published in this paper that the gelatin ion can dissociate electrolytically only as one of the two, either as a cation or as an anion but not as both simultaneously (except to a negligible degree), and that the hydrogen ion concentration of the solution alone determines in which of the two ways it dissociates. We shall also show that the isoelectric point is not only the point where the gelatin sends out as many H as OH ions but that it is the point where it apparently sends out neither; *i.e.*, where it cannot exist in an ionized condition at all.

II.

Our method of testing whether gelatin reacts with the cation or the anion of a neutral salt is based on the difference of the effects of univalent and bivalent ions upon gelatin. It was found in the writer's experiments on osmotic pressure, viscosity, alcohol precipitation, and swelling that a salt of the type Na_2SO_4 (univalent cation, bivalent anion) of a certain molecular concentration, *e.g.* M/128, has quantitatively and qualitatively the same effect upon gelatin (pH = 7.0) as a salt of the type NaCl at the molecular concentration M/64; *i.e.*, twice the molecular concentration of the Na_2SO_4.[4] This proves that only the cation of the salt and not the anion acts upon the gelatin. It was found, moreover, that all neutral salts with monovalent cation (Li, Na, K, NH_4) cause an increase in osmotic pressure, viscosity, alcohol number, and swelling of gelatin, while salts with a bivalent cation (Mg, Ca, Sr, Ba, Co, Mn) cause no such increase; and if a salt with bivalent cation is added in small quantity to a large quantity of a salt with univalent cation, the increase in osmotic pressure, viscosity, alcohol number, and swelling caused by the salt with univalent cation is inhibited (antagonistic salt action).[5] The same is

[4] Loeb, *J. Biol. Chem.*, 1918, xxxiv, 77, 489.

true for gelatin previously treated with NaOH. When such gelatin is subsequently treated with a salt with univalent cation its viscosity, osmotic pressure, swelling, etc., are increased, while when it is treated with a salt with bivalent cation this increase does not occur and its previous osmotic pressure, etc., are diminished. In all these cases the gelatin migrates as an anion, and the compounds formed are metal gelatinates where the metal is that of the alkali or salt used.

On the other hand, if gelatin has first been treated with an acid, *e.g.* HCl, gelatin chloride is formed which shows an increase in osmotic pressure, swelling, viscosity, and alcohol number. That such a gelatin (which is freed from the original acid by washing) reacts only with the anion of neutral salts is proved by the fact that a solution of a salt of the type $Ca(NO_3)_2$ (univalent anion, bivalent cation) influences gelatin chloride quantitatively and qualitatively like a salt of the type $NaNO_3$ (univalent anion, univalent cation) of half the molecular concentration; *e.g.*, M/128 $Ca(NO_3)_2$ acts like M/64 $NaNO_3$.[4] This is only possible if the anions of the neutral salt react with the gelatin chloride, while the cations have no effect, since otherwise the depressing effect of Ca mentioned before should be noticeable, which is not the case. If gelatin chloride freed from the excess of acid by washing is treated with a sufficiently high concentration of a neutral salt with univalent anion the osmotic pressure, swelling, viscosity, etc., of the gelatin increase after the salt is washed away. If, however, the anion of the salt used is bivalent, *e.g.* sulfate, oxalate, succinate, it causes less increase or an actual decrease of the osmotic pressure, viscosity, alcohol number, and swelling of the gelatin.

Hence by making use of the depressing effects of bivalent ions and the opposite effect of the univalent ions we can determine at which pH the gelatin begins to combine with the cation and ceases to combine with the anion of a neutral salt. Our experiments show that this critical point lies for gelatin at a hydrogen ion concentration 2.10^{-5} (pH = 4.7), *i.e.* the isoelectric point of gelatin, and not at the point of neutrality.

[5] All these effects were measured after the excess of the electrolyte was washed away (see footnote 1).

Our method was the same as in our previous experiments. 1 gm. of finely powdered gelatin was put into each of a series of beakers containing 100 cc. of a solution of HCl of various concentrations (M/8 to M/8192). This series served as a control. We then experimented with HCl solutions of different concentrations (M/8 to M/8192), each containing a certain salt (*e.g.* Na_2SO_4 or $Ca(NO_3)_2$) in the same concentration (M/16). The gelatin remained for 30 minutes in these solutions of a temperature of 20°C. The gelatin was then poured into cylindrical funnels of the same diameter, to allow the acid or acid-salt mixture to drain off, and the gelatin was then perfused three times with 25 cc. of distilled water of 10°C., and a fourth time with H_2O of 20°C.[6] The gelatin was then melted and made into a 1 per cent solution. Dialysis experiments, titration experiments, and determinations of pH by Sörensen and Clark's indicator method[7] made it probable that the solutions contained no acid except that due to hydrolytic dissociation of the gelatin salt formed; it was also possible to show that the neutral salt added to the HCl solution had been washed away.

The following physical qualities of the washed gelatin were ascertained:

1. The conductivity $\left(\frac{10{,}000}{\text{ohms}}\right)$, at 24°C.

2. The osmotic pressure expressed in mm. height of the 1 per cent gelatin solution in the manometer tube.

3. The total swelling expressed in mm. of the height of the cylindrical mass of gelatin.

4. The alcohol number; *i.e.*, the cc. of 95 per cent alcohol required to precipitate 5 cc. of 1 per cent gelatin solution at 20°C.[8]

The results are given in the form of curves in which the logarithms of the concentration of the acid used are the abscissæ and the above mentioned values for conductivity, osmotic pressure, swelling, and alcohol number, the ordinates. The pH of each gelatin solution was

[6] The water was used at these temperatures to prevent excessive swelling and a retardation of the process of filtration.

[7] We are obliged to Dr. Dernby for demonstrating to us the use of these methods.

[8] The viscosity of the solutions was determined in a special set of experiments and the viscosity curves ran parallel to those for osmotic pressure. They may therefore be omitted in this paper.

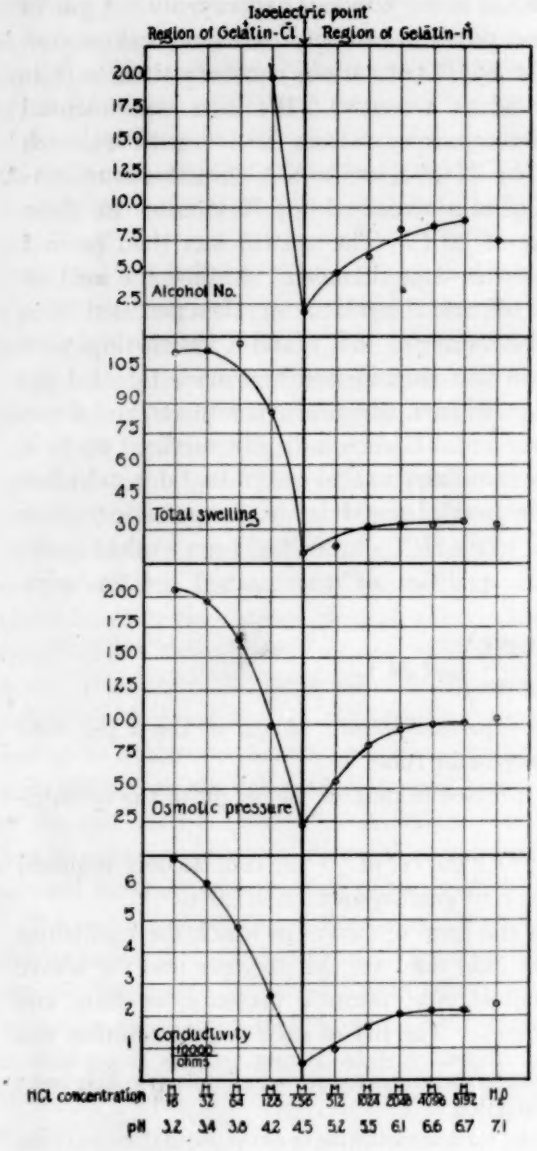

FIG. 1. HCl curves. Curves of the conductivity, osmotic pressure, swelling, and alcohol number of gelatin previously treated with various concentrations of HCl and then freed from excess of HCl by washing with water. On the axis of abscissæ are the logarithms of the HCl concentration used, and under each concentration is the pH of the 1 per cent gelatin solution in H_2O (made from the washed gelatin). The ordinates are the values for conductivity, osmotic pressure, swelling, and alcohol number for the gelatin. In the region of the isoelectric point (pH = 4.7) all the curves have a minimum. On the left of this point gelatin exists in the form of gelatin chloride (with high ionization), the curves rising more rapidly than on the right of the isoelectric point where the gelatin exists in the form of common gelatin which dissociates as a very weak acid. All four curves are nearly parallel.

ascertained and is put under the figures for the logarithms of the concentration.

We now will in turn discuss the four main groups of curves.

Fig. 1 gives the curves for gelatin treated for 30 minutes with HCl alone and then freed from the supernatant and intercapillary acid (with the exception of that formed by hydrolysis of gelatin chloride). The striking fact is that the conductivity, osmotic pressure, swelling, and alcohol number (and viscosity, though this curve is omitted) have all a minimum near a point where the hydrogen ion concentration pH = 4.7; *i.e.*, at about the isoelectric point as determined for gelatin solution by Michaelis on the basis of migration experiments. We may add that the transparency of the solution was also a minimum at the isoelectric point, where the solution was generally quite opaque after standing over night.

Though it was already known[3] that the viscosity and the swelling of gelatin are a minimum at pH = 4.7, our experiments for the first time furnish the proof that this is also the exact location of the minimum for conductivity, osmotic pressure, alcohol number, and transparency.

The second fact of significance is the sudden rise of the system of curves on either side of the isoelectric point. Since the conductivity curve is the direct expression of the degree of electrolytic dissociation existing in the gelatin solution (which had been freed from all ions except those formed by electrolytic and hydrolytic dissociation of the gelatin salt), these curves may be considered as a function of the degree of electrolytic dissociation of the gelatin.

Gelatin may exist in the form of non-ionized gelatin on both sides of the isoelectric point, but we shall endeavor to show that ionized gelatin exists in a different ionic state on the two sides of the isoelectric point. On the right, less acid side of the isoelectric point it can only exist as a negative ion (*e.g.* gelatin-$\overset{-}{H}$ or gelatin-$\overset{+}{Na}$, etc.), while on the left, more acid side of the isoelectric point it can only exist as a cation (*e.g.* gelatin-$\overset{+}{Cl}$ or gelatin-$\overset{+}{OH}$). When we study the curves for gelatin treated previously with HCl and freed from the excess of HCl by washing as described, the two branches of the curves on the two sides of the isoelectric point are asymmetrical (Fig. 1).

The left branches rise higher than the right ones on account of the fact that on the left side of the isoelectric point we have gelatin chloride which as a salt has a high dissociation constant and which dissociates into $\overset{+}{\text{gelatin}}$ and $\overset{-}{\text{Cl}}$, while on the right of the isoelectric point the curves are the expression of the electrolytic dissociation of common gelatin which dissociates like a very weak acid into $\overset{+}{\text{gelatin}}$ and $\overset{-}{\text{H}}$. Hence the limited rise of conductivity on the right and the considerable rise on the left of the isoelectric point.

The parallelism between the curve for conductivity and that for the other physical properties of the gelatin solution is so complete that the idea is unavoidable that the degree of conductivity, *i.e.* of the ionization of the gelatin, determines all the other physical properties of the gelatin solution; the pH only entering to the extent as it influences the possibility and character of the ionization of the gelatin. We shall see presently, however, that this parallelism between the conductivity and the curve for the other physical properties of the gelatin solution does not hold throughout but that in addition to the degree of electrolytic (and hydrolytic) dissociation still another variable enters. The main fact for us is the typical asymmetry of the two branches of the curve to the left and to the right of the isoelectric point, the branch on the left (the more acid side) being steeper and much higher than the branch on the right.

A comparison of the curves of Figs. 2, 3, and 4 with those of Fig. 1 will prove that gelatin when it ionizes will ionize on the right, less acid side of the isoelectric point as an anion, on the left, more acid side of the isoelectric point as a cation.

Fig. 2 represents the effect of the same concentrations of HCl as Fig. 1, except that each acid solution contained so much Na_2SO_4 as to make them all M/16 for this salt. We will call this system of curves the Na_2SO_4-HCl curves to distinguish them from the pure HCl curves represented by Fig. 1. The powdered gelatin was put for 30 minutes at 20° into these acid-salt mixtures and then freed from all the supernatant free acid and salt by the process of washing described in the writer's previous papers. If it is true that the isoelectric point is also a chemical turning point and that gelatin can ionize only as an

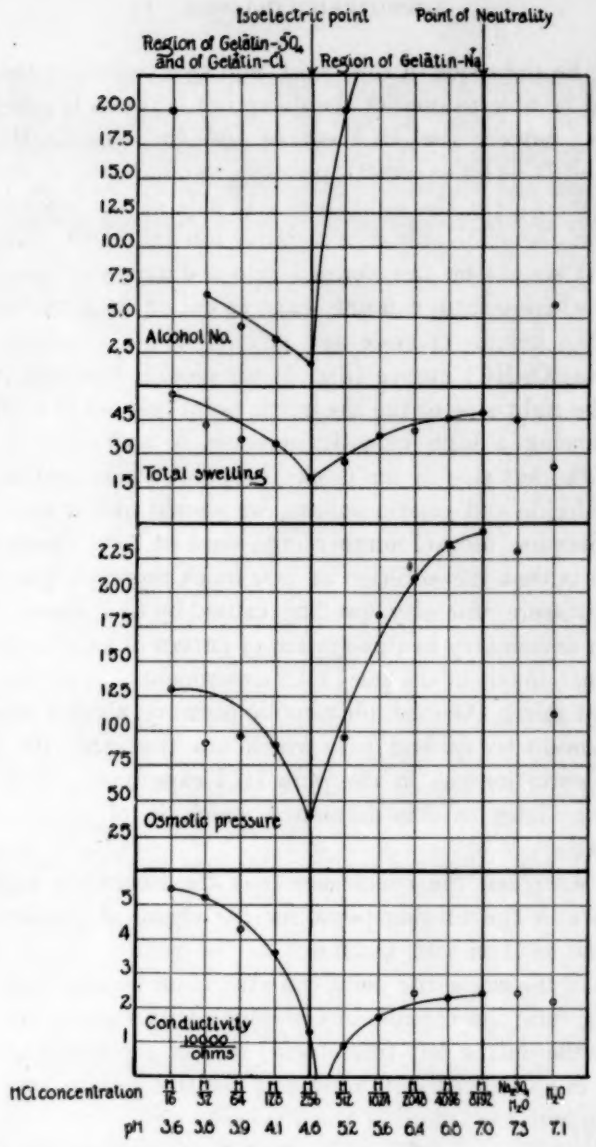

FIG. 2. Na_2SO_4-HCl curves. Isoelectric point and minimum again near pH = 4.7. Values for osmotic pressure, swelling, and alcohol number on right of isoelectric point, where gelatin exists as sodium gelatinate, higher than on left, where it exists as mixture of gelatin sulfate and gelatin chloride. The curves for osmotic pressure, swelling, and alcohol number are *not* parallel to the curve for conductivity.

anion on the right side of this point, and as a cation on the left side, we should be able to predict the difference between the two systems of curves, namely the HCl curves and the Na_2SO_4-HCl curves (Figs. 1 and 2), as far as osmotic pressure, swelling, and alcohol number are concerned. On the right, less acid side of the isoelectric point gelatin can dissociate only as a negative ion and hence, reacting with Na_2SO_4, it should be transformed into sodium gelatinate. On the left side, where gelatin can only exist as cation, in a mixture of HCl and Na_2SO_4 gelatin chloride and gelatin sulfate should be formed. In the Na_2SO_4-HCl curves (Fig. 2) we should, therefore, expect to find on the right side of the isoelectric point (pH > 4.7) sodium gelatinate, showing a high osmotic pressure, a high alcohol number, etc. On the left side of the isoelectric point where gelatin exists as gelatin chloride and gelatin sulfate, we should find lower values for osmotic pressure, alcohol number, etc., since we have shown in former experiments that the addition of SO_4 to Cl represses the increase in osmotic pressure, viscosity, swelling, caused by Cl. Hence we should expect an asymmetry in this system of curves of exactly the reverse kind of that found in the pure HCl experiment. A glance at Fig. 2 shows that this is the case, for osmotic pressure, alcohol number, but not for conductivity and pH, which are the same for the same HCl concentrations as in the pure HCl experiment (Fig. 1). We shall return later to this difference which is of great theoretical importance.

Hence we reach the conclusion that the isoelectric point of the gelatin is also the turning point for the chemical reaction between gelatin and neutral salt, inasmuch as the gelatin on the less acid, right side of the isoelectric point can exist in an ionized state only as a negative gelatin ion capable of combining with cations but not with anions, while on the left (more acid) side of the isoelectric point it can exist in an ionized state only as a positive gelatin ion capable of combining with the anions of a neutral salt.

This conclusion is confirmed by the experiments of the $Ca(NO_3)_2$-HCl series. In this series, represented by the curves of Fig. 3, the same concentrations of HCl from M/8 to M/4096 were used and each HCl solution contained so much $Ca(NO_3)_2$ as to make it M/16 in regard to $Ca(NO_3)_2$. If our theory is correct, the asymmetry of the

curves for osmotic pressure, swelling, and alcohol number should be the reverse for the $Ca(NO_3)_2$ series as that for Na_2SO_4. On the right side of the isoelectric point (for pH > 4.7) gelatin should exist in the form of Ca gelatinate which according to our previous observations has a low osmotic pressure, a low alcohol number, low viscosity, etc. On the left side of the isoelectric point it should exist in the form of gelatin chloride and nitrate in which form gelatin has a high osmotic pressure, swells considerably, has a high viscosity, and a high alcohol number. The forms of the curves actually found are represented in Fig. 3, and show that the facts agree with our theory.

Our theory finally demands that the two branches of the curve should become almost symmetrical in regard to the isoelectric point, if we add to the HCl a salt with univalent cation and univalent anion, *e.g.* NaBr. NaBr was added to the HCl solutions so as to make the solution M/16 in regard to this salt, and Fig. 4 gives the curves for gelatin treated with NaBr-HCl. It is obvious that the branches of the curves to both sides of the isoelectric point are much more symmetrical than was the case in the three other groups of curves. In this case we have on the right side of the isoelectric point Na-gelatinate, on the left a mixture of gelatin bromide and chloride. On both sides of the isoelectric point we have gelatin in combination with univalent ions, which as we have shown in our previous papers yield a high osmotic pressure, a high swelling, etc.

The experiments were repeated with other salts, $Mg(NO_3)_2$, Na_2 oxalate, etc., and our conclusions were confirmed. In the experiments described in this paper the reaction between the anions of the neutral salt with gelatin took place while the gelatin was in the acid solution; while the reaction between the cations of the neutral salt and gelatin could not take place while the gelatin was in the acid solution since all these concentrations were on the acid side of the isoelectric point of gelatin. This reaction must have taken place after the acid treatment and during the process of washing with H_2O when the gelatin previously treated with M/256 HCl had reached a pH > 4.7.

This conclusion is confirmed by the experiments in which the gelatin was first treated with acid, then freed from the excess of acid by perfusions with distilled water, and was then treated with a salt and

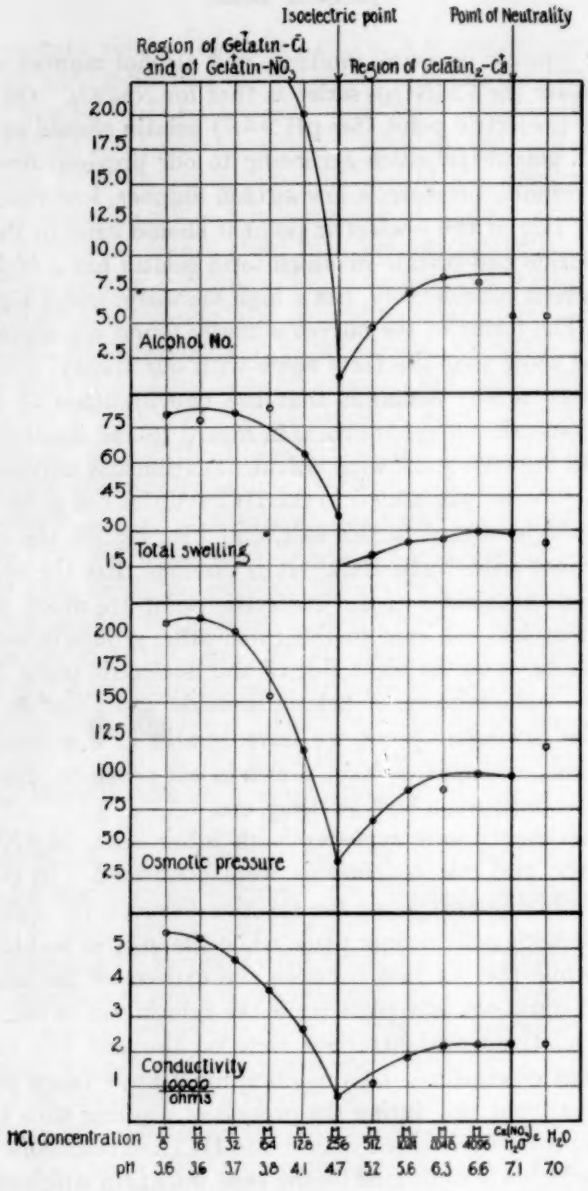

FIG. 3. $Ca(NO_3)_2$-HCl curves. Minima of all curves again at pH = 4.7 (isoelectric point). The branches of the curves for osmotic pressure, swelling, and alcohol number on the left, where gelatin exists as mixture of gelatin Cl and gelatin nitrate, higher than the branches on right of the isoelectric point, where gelatin exists as calcium gelatinate.

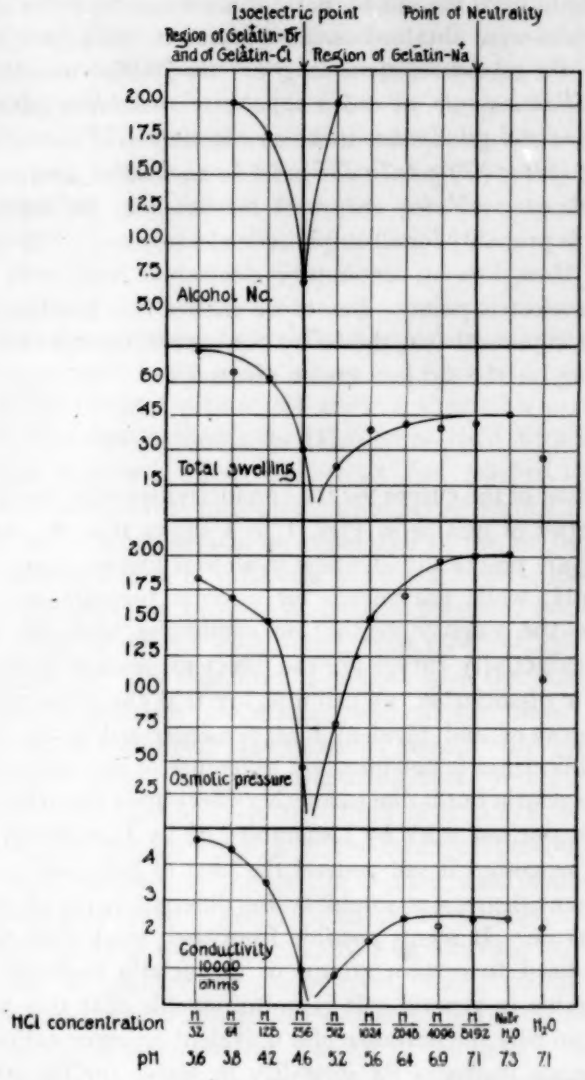

FIG. 4. NaBr-HCl curves. Minima again at pH = 4.7 (isoelectric point). Curves for osmotic pressure, swelling, and alcohol number almost symmetrical in regard to isoelectric point. On left side gelatin chloride and bromide, on right side sodium gelatinate.

then freed again from the salt by perfusion with water.[9] In this case the same results were obtained as those stated in this paper; namely: *When pH of the gelatin solutions is >4.7, the gelatin can exist in an ionized condition only as an anion capable of combining with cations only to form metal gelatinates, while on the more acid side of the isoelectric point (pH <4.7) gelatin can exist in an ionized form only as a cation capable of combining only with the anion of the neutral salt.* This law holds probably for all amphoteric electrolytes. The question then arises: How does an amphoteric electrolyte react with neutral salts at its isoelectric point? Before we answer this question we will point out an important fact shown by our curves, though this has no direct bearing on the subject under discussion.

III.

A comparison of the curves for the conductivities with those for the other properties of gelatin in Figs. 1 to 4 shows that the curves for conductivity are practically identical in all four figures as are also the values for pH; while the curves for osmotic pressure, etc., differ according to the valency of the ion combining with the gelatin. Since the conductivity curves are the direct expression of the degree of electrolytic dissociation, we can now say that the difference in the effect of univalent and bivalent ions demonstrated in the writer's previous publications is not merely a difference of the degree of ionization of the gelatin but is due to another effect upon the gelatin molecule. The important work by Langmuir and by Harkins on surface tension has suggested if not proved the idea of polarized molecules and of different degrees of solubility for different parts of the same organic molecule. It seems possible from their work that the solubility is confined to certain groups of the gelatin molecule or the protein molecule in general. It is quite possible that this will ultimately explain why the entrance of a univalent anion or cation into a gelatin molecule increases its solubility in water (or its attracting power for water molecules) and with this its osmotic pressure, alcohol number, viscosity, etc., while the entrance of a bivalent ion (com-

[9] In order not to increase the bulk of this paper we may be pardoned for omitting the details of these experiments.

bining possibly with two or more molecules of gelatin) will not cause or may even prevent such an increase, without altering the conductivity of the gelatin solution. The physical qualities of a gelatin solution depend, therefore, aside from the degree of its electrolytic dissociation and its hydrogen ion concentration upon a constitutional factor varying with the valency of the ion with which the gelatin is in combination. We intend to return to this question in a later publication.

IV.

The writer was curious to find out how gelatin reacts with neutral salts when brought to the isoelectric point. This was done by treating gelatin for 30 minutes with M/256 acetic acid or with M/256 or M/512 HCl, etc., and then perfusing the gelatin repeatedly with 25 cc. of H_2O in the way described. That the gelatin had reached the isoelectric point was ascertained by measurements of pH, of conductivity, osmotic pressure, etc. When gelatin on either side of the isoelectric point is treated with M/4 or M/8 NaCl or any other salt with univalent anion or cation, it shows an increase in osmotic pressure, swelling, alcohol number, after the salt is washed away; when, however, gelatin rendered isoelectric in the way described is treated with the same solution of NaCl, all its physical properties, conductivity, osmotic pressure, swelling, alcohol number, transparency, remain unaltered after the salt is washed away. It is immaterial whether we treat such gelatin with NaCl, Na_2SO_4, $CaCl_2$, or any other neutral salt; and it is also immaterial whether we use high or low concentrations of these salts; the physical qualities of the gelatin remain unaltered. Numerous experiments were made in which the nature of the acid, the concentrations, the number of washings, etc., used to reach the isoelectric point varied, but all yielded the same result, which means that *at the isoelectric point gelatin is chemically inert*, incapable of reacting with neutral salts. When, however, the pH of the gelatin deviates only slightly from that of the isoelectric point by becoming either $>$ or <4.7, the neutral salts react with gelatin in the way described in this and previous papers.

This is exactly what our theory demands, according to which gelatin can dissociate electrolytically only as an anion when pH is $>$ 4.7, as

a cation when pH is <4.7. Hence it can dissociate as neither when pH = 4.7. Not being able to dissociate electrolytically, it cannot react chemically with the ions of a neutral salt, as was found to be the case.

This inability to dissociate electrolytically and hence to react with neutral salts at the isoelectric point should be a common property of all amphoteric electrolytes and this seems to be the case. While this note goes to press a paper has appeared by Van Slyke and Baker,[10] containing the following sentence. "At the isoelectric point casein combines with neither acid nor base;" *i.e.*, with neither cation nor anion.

V.

The facts shown thus far support the idea that amphoteric electrolytes dissociate in a different way on the two sides of the isoelectric point; on the less acid side of that point they can ionize only as anions capable of combining with the cations of neutral salts, but not with their anions; on the acid side of the isoelectric point they can ionize only as cations capable of combining with the anion of a neutral salt, but not with its cation. The question then arises: What will happen when we bring a salt like sodium gelatinate in which gelatin is an anion on the acid side of the isoelectric point? Our theory demands that sodium gelatinate produced by treating gelatin with NaOH should not continue to exist when pH<4.7, and that in this case gelatin should behave as if it had not been treated with NaOH. This is what actually happens. The writer made this experiment some time before he had arrived at the theory published in this paper. He had undertaken the experiment in the expectation that gelatin first treated with alkali and then with acid should show a superposition of the effects of both treatments. 1 gm. of finely powdered gelatin was put for 30 minutes at 20°C. into each of a series of beakers all containing M/32 NaOH. After this, each powder was put on a filter, the alkali was allowed to drain off, and each filter was perfused four times with 25 cc. of H_2O to remove the last traces of NaOH held in the capillary spaces (except the NaOH formed by the hydrolytic dissociation of the sodium gelatinate). Then each filter was perfused three

[10] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 147.

times with 25 cc. of a HCl solution which varied for the different filters from M/8 to M/8192, and after this each filter was perfused four times with H_2O to remove the HCl not bound by the gelatin.

The result is given in the curves of Fig. 5 for the conductivity, osmotic pressure, swelling, and alcohol number. The curves show a marked minimum at about pH = 4.8; *i.e.*, the isoelectric point. The appearance of this low minimum of the values for conductivity, osmotic pressure, swelling, etc., characteristic of the isoelectric point, was at first a surprise and a puzzle to the writer. On the right side the curve of osmotic pressure rises to a value of 125 to 150 mm. which corresponds to the value for gelatin treated with M/32 NaOH and washed ten times with H_2O (without any subsequent treatment with HCl). On the acid side of the isoelectric point the curve of osmotic pressure rises to that point which corresponds to the effect the same concentration of HCl has on gelatin not previously treated with alkali. Hence the result of the acid treatment is on the left, more acid side from the isoelectric point, exactly the same as when we use gelatin not previously treated with M/32 NaOH and apply to it the same treatment with HCl as in this experiment; while on the right, less acid side of the isoelectric point, it is the same as if the gelatin had been treated with NaOH of increasing concentrations.

This permits of only one explanation, which in the light of the experiments published in the preceding parts of this paper is as follows. Sodium gelatinate, or perhaps more correctly a negative gelatin ion, cannot continue to exist except at hydrogen ion concentrations lower than that defining the isoelectric point. This means that in the acid solutions all or most of the sodium gelatinate must have given off its sodium ions and must have been retransformed into the weak gelatin acid; or some other constitutional (tautomeric?) change must have occurred in the gelatin molecule on the acid side of the isoelectric point. On the alkali side of the isoelectric point the sodium gelatinate could and did continue to exist.

This experiment was repeated with different concentrations of acids and alkalies, all confirming our conclusions. The experiment was also reversed, *i.e.* the gelatin was first treated with acid and then with alkali, and again our theory was confirmed.

It is of interest to see our view confirmed also by another set of facts. It has long been known that proteins and probably ampho-

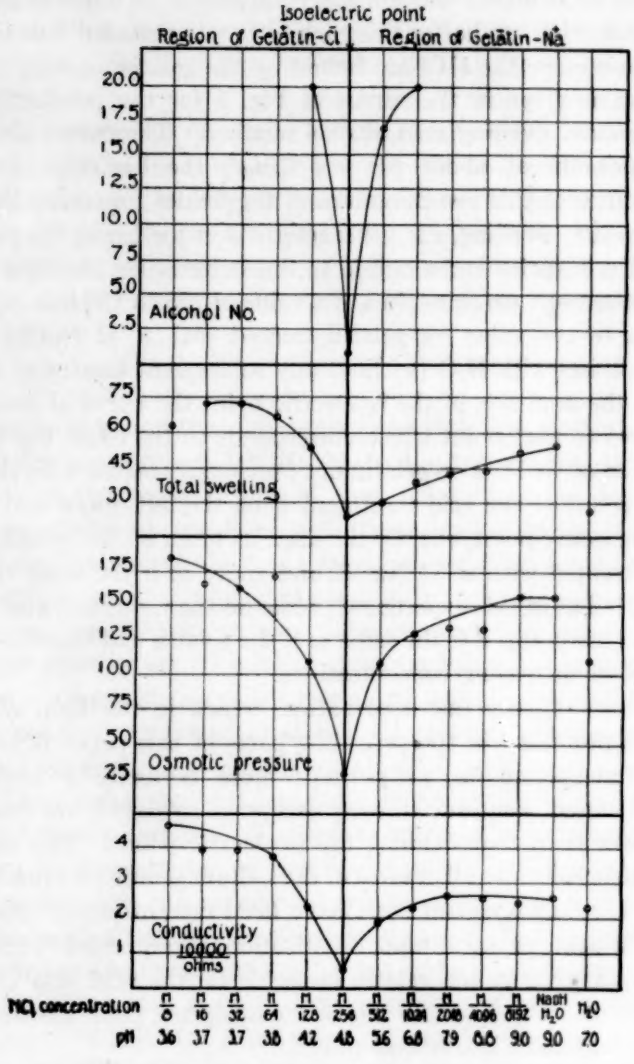

FIG. 5. M/32 NaOH followed by HCl treatment. Minima at pH = 4.8 (isoelectric point). Branches of curves on left side almost identical with those of Fig. 1 (where HCl alone was applied). On the left side of isoelectric point gelatin exists as gelatin chloride, on the right side as sodium gelatinate.

teric electrolytes in general stain with acid dyes in an acid but not in an alkali solution, and with basic dyes only in an alkaline solution. This agrees with our theory that an amphoteric electrolyte like gelatin can exist as an anion capable of combining with a basic dye (*e.g.* neutral red) only on the basic side of its isoelectric point, and that gelatin can exist as a cation capable of combining with acid dyes (*e.g.* acid fuchsin) only on the acid side of its isoelectric point. Our theory further demands that gelatin when stained with neutral red should give off its dye in solutions on the acid side of its isoelectric point, since on this side of the isoelectric point gelatin can no longer exist as an anion, while gelatin stained with acid fuchsin should give off its dye in solutions with a pH >4.7, since here the gelatin can no longer exist as a cation. All this turns out as our theory demands. The writer intends to make further experiments on this problem, but the facts thus far observed support our theory that when dissociating electrolytically an amphoteric electrolyte is stable as an anion only on the basic side of its isoelectric point, and as cation on the acid side of its isoelectric point. Of course, in a non-ionized condition gelatin may exist at any pH.

Another apparent support of our theory lies in the old observation made by Hamburger[11] on the absorption of Cl and SO_4 by the red blood corpuscles from serum when the CO_2 of the blood rises. In this case it is probably simply a question of raising the pH of the blood above that of the isoelectric point for hemoglobin, which is near the point of neutrality. The whole problem of the retention and excretion of electrolytes by the body may become intelligible from the fact proved in this paper that proteins or amphoteric electrolytes in general can exist as cations only (and have a tendency to become cations) on the more acid side of the isoelectric point, while they can exist as anions only (and have a tendency to become anions) on the less acid side of their isoelectric point.

[11] Hamburger, H. J., and van Lier, G. A., *Arch. Physiol.*, 1902, 492. Hamburger, *Biochem. Z.*, 1918, lxxxvi, 309. See also, Hasselbalch, K. A., and Warburg, E. J., *ibid.*, 1918, lxxxvi, 410.

SUMMARY.

1. It has been shown in this paper that while non-ionized gelatin may exist in gelatin solutions on both sides of the isoelectric point (which lies for gelatin at a hydrogen ion concentration of $C_H = 2.10^{-5}$ or pH = 4.7), gelatin, when it ionizes, can only exist as an anion on the less acid side of its isoelectric point (pH > 4.7), as a cation only on the more acid side of its isoelectric point (pH < 4.7). At the isoelectric point gelatin can dissociate practically neither as anion nor as cation.

2. When gelatin has been transformed into sodium gelatinate by treating it for some time with M/32 NaOH, and when it is subsequently treated with HCl, the gelatin shows on the more acid side of the isoelectric point effects of the acid treatment only; while the effects of the alkali treatment disappear completely, showing that the negative gelatin ions formed by the previous treatment with alkali can no longer exist in a solution with a pH < 4.7. When gelatin is first treated with acid and afterwards with alkali on the alkaline side of the isoelectric point only the effects of the alkali treatment are noticeable.

3. On the acid side of the isoelectric point amphoteric electrolytes can only combine with the anions of neutral salts, on the less acid side of their isoelectric point only with cations; and at the isoelectric point neither with the anion nor cation of a neutral salt. This harmonizes with the statement made in the first paragraph, and the experimental results on the effect of neutral salts on gelatin published in the writer's previous papers.

4. The reason for this influence of the hydrogen ion concentration on the stability of the two forms of ionization possible for an amphoteric electrolyte is at present unknown. We might think of the possibility of changes in the configuration or constitution of the gelatin molecule whereby ionized gelatin can exist only as an anion on the alkaline side and as a cation on the acid side of its isoelectric point.

5. The literature of colloid chemistry contains numerous statements which if true would mean that the anions of neutral salts act on gelatin on the alkaline side of the isoelectric point, *e.g.* the alleged effect of the Hofmeister series of anions on the swelling and osmotic pressure of common gelatin in neutral solutions, and the statement

that both ions of a neutral salt influence a protein simultaneously. The writer has shown in previous publications that these statements are contrary to fact and based on erroneous methods of work. Our present paper shows that these claims of colloid chemists are also theoretically impossible.

6. In addition to other physical properties the conductivity of gelatin previously treated with acids has been investigated and plotted, and it was found that this conductivity is a minimum in the region of the isoelectric point, thus confirming the conclusion that gelatin can apparently not exist in ionized condition at that point. The conductivity rises on either side of the isoelectric point, but not symmetrically for reasons given in the paper. It is shown that the curves for osmotic pressure, viscosity, swelling, and alcohol number run parallel to the curve of the conductivity of gelatin when the gelatin has been treated with acid, supporting the view that these physical properties are in this case mainly or exclusively a function of the degree of ionization of the gelatin or gelatin salt formed. It is pointed out, however, that certain constitutional factors, *e.g.* the valency of the ion in combination with the gelatin, may alter the physical properties of the gelatin (osmotic pressure, etc.) without apparently altering its conductivity. This point is still under investigation and will be further discussed in a following publication.

7. It is shown that the isoelectric point of an amphoteric electrolyte is not only a point where the physical properties of an ampholyte experience a sharp drop and become a minimum, but that it is also a turning point for the mode of chemical reactions of the ampholyte. It may turn out that this chemical influence of the isoelectric point upon life phenomena overshadows its physical influence.

8. These experiments suggest that the theory of amphoteric colloids is in its general features identical with the theory of inorganic hydroxides (*e.g.* aluminum hydroxide), whose behavior is adequately understood on the basis of the laws of general chemistry.

Addendum. Since this paper went to print the writer has been able to show by volumetric analysis that the view expressed in this paper is correct; namely, that on the alkaline side from the isoelectric point gelatin combines with the cation of a neutral salt,

while on the more acid side from the isoelectric point it combines with the anion of a neutral salt. The curve for the amount of cation or anion in combination with the gelatin runs parallel with the curves for osmotic pressure, swelling, etc. The new experiments will be discussed in the second number of this *Journal*.

A THEORY OF THE MECHANISM OF DISINFECTION, HEMOLYSIS, AND SIMILAR PROCESSES.

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(Received for publication, July 25, 1918.)

The apparent course of such processes as hemolysis is determined by the rate of change in the number of living cells which have undergone some definite alteration, such as laking or loss of viability. A great deal of confusion has arisen from the attempts of various investigators to deduce from the observed course of disinfection and hemolysis the nature of their fundamental reactions. Processes of this kind have often been looked upon as due to reactions of a monomolecular type, solely because of a superficial resemblance between the curves expressing the rate of progress of the two phenomena.

This paper is a critical discussion of the part played by the physicochemical process or group of processes leading to death, laking, and similar effects in determining progressive changes in the number of individual cells succumbing in successive units of time to the action of the deleterious agent. These physicochemical processes in the protoplasm may, for the sake of brevity, be termed the "fundamental reaction;" by the "course of the process" we shall understand the time curve of any process like hemolysis or disinfection. The subject is treated in four sections dealing with (1) the evidence that the degree of hemolysis is determined by the number of cells which are laked, and that this depends on the fact that individual cells possess different degrees of resistance; (2) the influence taken singly and in combination, of changes in the variation curve of resistance and of progressive changes in the velocity of the fundamental reaction; (3) the hypotheses advanced to account for the observed course of disinfection, hemolysis, and like processes; (4) the interpretation and significance of the time curves of such processes.

I.

If erythrocytes are suspended in an indifferent medium and subjected to a brief radiation from a mercury vapor arc in quartz, or if they are suspended in an appropriate dilution of serum acting in conjunction with a specific antibody, there ensues a gradual liberation of hemoglobin. In both cases this process begins at a rather slow rate which gradually increases, passes through a maximum, and then gradually falls off until it becomes comparable with the rate of spontaneous laking. (See Table I.)

TABLE I.

Observed Course of Hemolysis; 100 Per Cent Signifies Completion.

10 min. exposure to Hg. arc in quartz.		Specific serum hemolysis.						
		Experiment 26, complement 0.2 per cent.			Experiment 20, complement 0.67 per cent.		Experiment 20, complement 0.23 per cent.	
Time after radiation.	Colorimetric estimation.	Time.	Color.	Cell count.	Time.	Color.	Time.	Color.
min.	*per cent*	*min.*	*per cent*	*per cent*	*min.*	*per cent*	*min.*	*per cent*
5	1	0	0	0	0	0	0	0
12	2	3	3	—	10	65	30	5
19	3	6	5	12	20	94	60	28
37	5	10	10	—	40	100	120	38
55	12	14	21	45			210	45
88	29	20	30	—				
145	55	28	56	55				
205	72	41	67	73				
295	87	56	74	—				
660	100	85	81	79				
		112	81	—				
		155	82	82				
		1,320	100	100				

Plotting as ordinates the amount of hemoglobin liberated, and as abscissæ the times of sampling, one obtains asymmetrical sigmoid curves such as those shown in Fig. 1. The gradual retardation and final apparent equilibrium is either due to exhaustion or inactivation of the lytic agent, or if the concentration of the lytic agent (photoproduct or serum) is increased above that necessary to produce complete hemolysis in a few hours, the process ceases because of the ex-

haustion of the supply of cells. (In the latter case we may conceive that the fundamental reaction is still proceeding at a relatively rapid rate even in the most resistant cells at the time when they succumb.)

Enumeration of the red blood cells visible in a given volume of suspension at various stages of hemolysis shows that the amount of hemoglobin liberated is very nearly proportional to the number of cells which have lost their pigment (Table I and Fig. 1). Handovsky[1] finds a similar parallelism between the disappearance of cells and the liberation of hemoglobin when erythrocytes are partially hemolyzed by saponin in low concentrations.

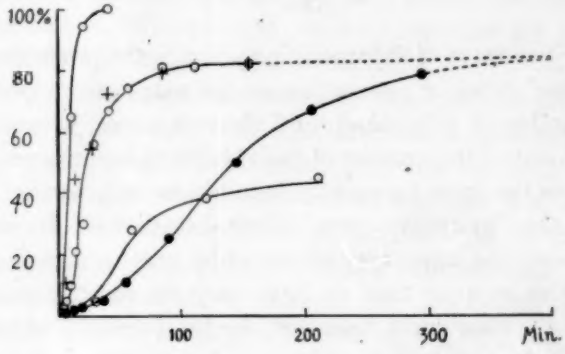

FIG. 1. The time curve of hemolysis produced by ultra-violet radiation (solid circles), and by various dilutions of complement acting on specifically sensitized cells (open circles); the degree of hemolysis was colorimetrically determined. The crosses represent the degree of hemolysis as determined by cell counts during the course of the process represented by the adjoining curve. The ordinates represent the amount of hemolysis in per cent of completion, and the abscissæ, time in minutes.

The course of hemolysis depends therefore on the relative number of red blood cells having in different degrees the power to resist the action of the lytic agent. For since the amount of hemoglobin liberated is always proportional to the number of erythrocytes which have disappeared we must regard the observed course of hemolysis as the summation of the laking of individual cells at varying times after they are subjected to the condition leading to hemolysis. A few relatively

[1] Handovsky, H., *Arch. exp. Path. u. Pharmakol.*, 1912, lxix, 412.

fragile cells are laked almost immediately, the resistant ones survive for many hours, but most of the cells succumb during an intermediate period when the observed rate of hemolysis is at a maximum. When hemoglobin finally begins to diffuse from a given erythrocyte, the process is so quickly completed that it may ordinarily be regarded as instantaneous. It is obvious that an increase in the relative number of, for example, the more fragile cells would accelerate the earlier stages of hemoglobin liberation, while a decrease would produce a corresponding retardation.

II.

There are two ways of expressing graphically the progress of hemolysis: the time curve or course, whose ordinates are proportional to the total number of cells laked; and the rate curve, whose ordinates are proportional to the number of individuals laking in a unit of time. The former is the more frequently found in hemolytic studies, while the latter is the "mortality curve" of vital statistics. If one of these curves is known the other may be found by graphical methods. For instance, let us suppose that we have only the mortality curve: the ordinates of the time curve represent the total number of individuals having insufficient resistance to survive beyond the indicated time; the area enclosed by the mortality curve corresponds to the total number of individuals; the area enclosed by the ordinate at any point on the x axis and the part of the mortality curve to the left of the ordinate, is proportional to the number of cells having less than the indicated resistance, and is therefore also proportional to the corresponding ordinate of the time curve. If the mortality curve is plotted on suitable coordinate paper these areas may easily be estimated with sufficient accuracy, ordinates proportional to them erected at the corresponding points on the x axis, and their tops connected by a smoothed curve. This will be the time curve, and, since the process of finding it is a process of integration, it is at the same time an integral curve.

If we have only the time curve, or integral, it is easy to see that the rate of hemolysis, that is the number of cells laking in a unit of time, is represented by the slope of the time curve; the steeper the time

curve, the more individuals are succumbing per minute. The ordinates of the mortality curve are then at any time proportional to the steepness of the time curve. The slope or steepness is best found by plotting the time curve on suitable coördinate paper, drawing straight lines tangent to it at several points, and counting the number of squares passed over vertically by such lines for a given number passed over horizontally. This ratio is the trigonometric tangent of the slope of the time curve. If we draw ordinates proportional to these ratios at corresponding points on the x axis, and connect the tops of these ordinates by a smooth curve, we shall obtain the rate or mortality curve. Since the process by which we have gotten the rate curve is a graphic method of differentiation, we may consider the curve to be the differential of the time or integral curve.[2]

Under certain conditions the mortality curve may be identical with the variation or frequency curve of individual resistance. In the following paragraphs the relationship between the time curve and the variation curve is considered, starting with the simplest imaginable conditions, then varying the frequency curve alone, the course of the fundamental reaction alone, and finally both together.

If the rate of hemolysis is uniform, its time curve would be a sloping straight line (the integral curve, *a*, Fig. 2), while since its tangent or slope is the same at every point, the differential or mortality curve would be a straight line parallel to the axis of the abscissæ (*b*, Fig. 2); this condition could be expressed by the differential equation

$$\frac{dn}{dt} = k$$

where n is the number of cells, t the time, and k is a constant.

If we now assume the cells to be divided into classes differing from each other by one unit of resistance (r) (defining as a unit of resistance the power to resist one unit of fundamental reaction; *e.g.*, the formation of one mol of toxic substance), and further assume that the fundamental reaction is proceeding at a uniform rate, the differential equation may be replaced by the equation of a frequency curve, or

[2] This relationship has been suggested by Davey (*J. Exp. Zool.*, 1917, xxii, 573), in connection with curves representing the per cent of death occurring among flour beetles (*Tribolium confusum*) at various times after X-radiation.

variation curve in which all classes are equal in respect to number of individuals. This equation is $y = y_0$, where y_0 is the number of individuals in any one of the equal classes which is arbitrarily selected as the mode, and y is the number of individuals having x units of resistance less or more than the mode. It is obvious that in this particular case any class may be the mode. When the equation assumes a different form so that the classes are unequal (as in the ordinary curves found in biological work) and in consequence y varies, y_0 is the maximum ordinate of the variation curve.

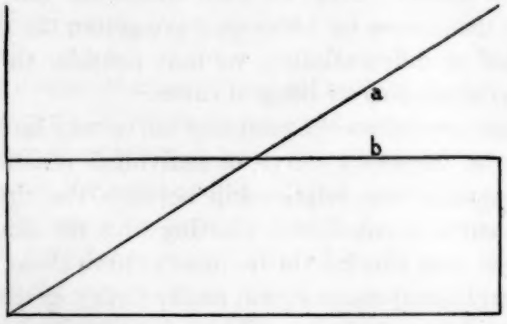

FIG. 2. The relation between the time curve (*a*) of a reaction proceeding at a constant rate, and the curve of the differential equation (*b*). The ordinates represent the extent to which the reaction has proceeded (*a*) or the rate at which it is proceeding (*b*), and the abscissæ represent time.

In order to understand the effect of changes in the shape of such variation curves, that is of changes in the relative number of cells having different degrees of resistance, let us retain the assumption that the fundamental reaction proceeds at a uniform rate; for this allows us to think of the variation curve (abscissæ = resistance) as being at the same time the differential of the time curve (abscissæ = time). Now suppose the variation curve to have the form (*a*, Fig. 3) commonly found in biological statistics, of a "skew frequency curve of limited range" whose equation according to Pearson[3] is

$$y = y_0 \left(1 + \frac{x}{x_1}\right)^{kx_1} \left(1 - \frac{x}{x_2}\right)^{kx_2} \qquad (1)$$

where k is a constant, and x_1 and x_2 the number of degrees of resist-

[3] Pearson, K., *Phil. Tr.*, A, 1895, clxxxvi, 343.

ance less and more, respectively, than the mode, possessed by the most fragile and most resistant classes. Here $x_1 + x_2$ is the total range of resistance of all the cells. A curve of this type (*a*) and its integral, or time curve, (*b*) are shown in Fig. 3; the time curve has a form not unlike that of the curves for the course of hemolysis.

If we suppose, with some investigators, that the resistance of the cells varies around an average from which it does not deviate to an extent sufficient to influence the course of the process, we must consider all the cells to be in a single class with respect to resistance.

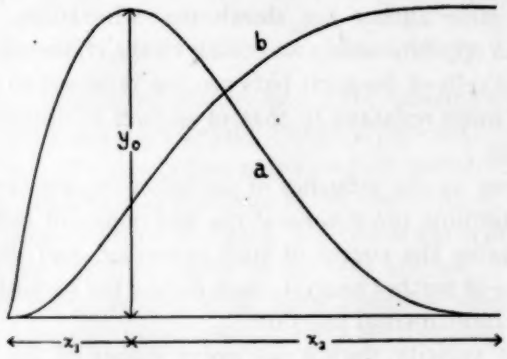

FIG. 3. (*a*) variation curve whose equation is

$$y = y_0 \left(1 + \frac{x}{x_1}\right)^{kx_1} \left(1 - \frac{x}{x_2}\right)^{kx_2}, \text{ where } x_1 = 2,\ x_2 = 6,\ k = 0.5.$$

If (*a*) is considered as the curve of a differential equation, (*b*) is the curve of the corresponding integral. The ordinates of (*b*) are proportionate at any position on the *x* axis (abscissæ) to the area to the left of the ordinate at that position, and under the curve (*a*).

The frequency curve will then be so narrow as to be approximately a straight line normal to the axis of the abscissæ at some point. Its integral, which is the time curve of the process, will follow the axis of abscissæ to this point, and then rise perpendicularly to its ultimate height. In other words, if the process were hemolysis, all the cells would lake at the same instant. This conclusion may be avoided, theoretically at least, by making one of the special assumptions which are discussed below.

As a matter of fact the exact shape of the frequency curve—that is the relative abundance of cells having different degrees of resistance to any given lytic agent—is not of general significance, for it depends on the condition and previous treatment of the animal from which the cells are secured, on the nature of the lysin, etc. For example, Handovsky[1] has shown that during the regeneration of erythrocytes following artifically induced anemia there are two groups of erythrocytes, one of which has a higher average resistance to hemolysis by saponin than the other; differentiation of curves representing the course of the hemolysis of such blood cells gives a bimodal variation curve. The same author has shown that alterations in resistance may appear in opposite senses according to the choice of lytic agents; *e.g.*, the blood cells of dormant bats are less resistant to the action of saponin, but more resistant to that of sodium hydroxide than those of active bats.

We may sum up the influence of variations of resistance by saying that they determine the general shape and points of inflection of the curves expressing the course of such processes, and that, therefore, in the absence of further analysis such curves tell us nothing as to the nature of the fundamental reaction.

Changes in velocity during successive stages of the fundamental reaction will obviously produce corresponding changes in the time required to produce a given degree of hemolysis, and will therefore alter the shape of the time curve. We have seen that when the equation of the variation curve of resistance is of the form

$$y = y_0$$

and the velocity of the fundamental reaction is constant, the time curve of the reaction is identical with that of the process as a whole. A moment's reflection will show that, whatever the course of the fundamental reaction, so long as the variation curve remains of this form the same identity will appear. If for example the fundamental reaction is monomolecular, the course of the process will appear monomolecular.

The relative length of time required for the process to reach any given stage (*i.e.* the abscissa of the time curve for any given ordinate) will be greater for the same initial velocity in the case of a monomo-

lecular reaction than in the case of a reaction proceeding at a constant rate, and the lengths will be still greater for reactions of higher orders.

The relative times, t_1 and t_0, required for two reactions with the same initial velocity to reach the same stage, when one is monomolecular and the other has a constant velocity, may be easily derived from the equations of the two time curves. The expression is

$$\frac{t_1}{t_0} = \frac{1}{x} ln \frac{a}{a-x}$$

where ln is the logarithm to the base e ($2.3026 \log_{10} \frac{a}{a-x}$ may advantageously be used in place of $ln \frac{a}{a-x}$), and where a is the initial amount of the reacting substance, and x the amount transformed at any given stage; a and x are usually stated in per cent.

If we compare the rates of hemolysis which would result from the action of two fundamental reactions of different orders, we find that the relative time required to reach a given stage will be the same regardless of the variation curve of the cells affected; if the shape of the variation curve is given by some equation other than $y = y_0$ (which gives identical time curves for the fundamental reaction and for the process as a whole), we can still calculate the relative lengths of time required for the process to reach a given stage, according to the order of the fundamental reaction. If the fundamental reaction is monomolecular or of a higher order, we must use as abscissæ distances which are as much greater than those of the time curve when the fundamental reaction has a constant velocity, as is indicated by calculations like that given above. This is most easily done if it is assumed that the hemolysis and the fundamental reaction reach completion simultaneously, but may still be calculated for a known excess or deficiency of the lytic agent. In Fig. 4 are given the courses which would be assumed by a process taking place in a group of cells, the equation of whose variation curve was

$$y = y_0 \left(1 - \frac{x^2}{x_1^2}\right)^{kx_1}$$

if the fundamental reaction were (*a*) proceeding with a constant velocity; (*b*) proceeding according to the law of monomolecular

reactions. Here again we find a curve *b* which simulates the observed curves for the course of hemolysis. We shall return to this point later on.

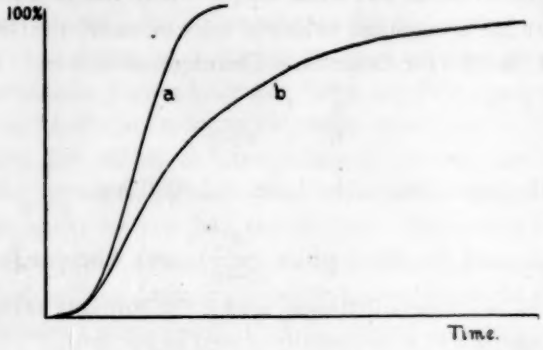

FIG. 4. The time curve of a process occurring in a group of individuals having a variation curve with the equation $y = y_0\left(1 - \frac{x^2}{x_1^2}\right)^{kx_1}$, if the fundamental reaction proceeds at a uniform velocity (*a*), and if it proceeds according to the law of monomolecular reactions (*b*). Here $x_1 = 1.5$ and $k = 2$. The ordinates represent degree of completion of the process, and the abscissæ, time.

III.

Various investigators have reported that bacteria in the presence of disinfectants die at a rate which is at any given time proportional to the number of organisms then surviving.[4-9] This condition would be described by the equation

$$\frac{dn}{dt} = kn_t, \text{ or } n_t = n_0 e^{-kt}$$

where n_0 and n_t are the number of bacteria at the beginning of the experiment and at t units of time thereafter, and e is the base of the natural system of logarithms. This is the equation of the mono-

[4] Madsen, T., and Nyman, M., *Z. Hyg.*, 1907, lvii, 388.
[5] Chick, H., *J. Hyg.*, 1908, viii, 92.
[6] Chick, *J. Hyg.*, 1910, x, 237.
[7] Phelps, E. B., *J. Infect. Dis.*, 1911, viii, 27.
[8] Eijkman, C., *Folia Microbiol.*, 1912, i, 359.
[9] Reichenbach, H., *Z. Hyg.*, 1911, lxix, 171.

molecular reaction isotherm. Similar relationships have been reported in the case of hemolysis, which is a process in many ways similar to disinfection;[10–14] although in this case a so called "induction period," often of considerable length (Dreyer and his coworkers) intervenes before the hemolysis appears to follow the course of a monomolecular reaction. In both hemolysis and disinfection a large number of single living cells are exposed to the action of an agent which ultimately induces in the cell some change which we can detect; in one case loss of power to reproduce; in the other, loss of a pigment; in both cases there is great variation in the length of time required to bring about the critical change in different cells. Harvey[15] considers the equation for monomolecular reactions to be applicable to the loss of motility suffered by cells of *Chlamydomonas* subjected to the action of hydrochloric acid in great dilution; while Darwin and Blackman, according to Arrhenius,[16] saw the same relationship when they allowed various killing agents to act on seeds. These citations, while by no means complete, will suffice to indicate the wide range of phenomena which have been studied from this point of view.

Some authors, like Dreyer and his coworkers, have not attempted to attribute the apparent analogy of these processes with monomolecular reactions to any single relationship, but some other authors have devoted to this phenomenon a great deal of discussion which does not seem to have been based upon a comprehensive knowledge of the subject, for their attempts to explain the analogy will not bear criticism, and none of their criticisms are entirely satisfactory. The difficulty is largely due to failure to see the necessary consequences of uniformity in resistance, or to disregard of the possible influence of progressive changes in the velocity of the fundamental reaction.

[10] Arrhenius, S., and Madsen, T., *Z. physik. Chem.*, 1903, xliv, 33.

[11] Henri, V., *Compt. rend. Soc. biol.*, 1905, lvii, 37.

[12] von Liebermann, L., and von Fenyvessy, B., *Z. Immunitätsforsch., Orig.*, 1912, xii, 417.

[13] Salomonsen, C. J., and Dreyer, G., *Compt. rend. Acad.*, 1907, cxliv, 999.

[14] Dreyer, G., and Hanssen, O., *Compt. rend. Acad.*, 1907, cxlv, 371.

[15] Harvey, H. W., *Ann. Bot.*, 1909, xxiii, 181.

[16] Arrhenius, S., Quantitative laws in biological chemistry, London, 1915, interprets in this way the experiments of Darwin, N., and Blackman, A., *Rep. 78th Meeting, 1908, Brit. Assn. Adv. Sc.*, 1909, 902.

Madsen and Nyman,[4] who were the first to notice the analogy, recognized the fact that variability among the cells was a factor to be reckoned with, but appear nevertheless to have regarded their curves as expressions of the average rate of change in the individual cells. We have seen that it is impossible, without special assumptions, to account for the phenomenon on this basis. Miss Chick,[5] working independently, secured data like those of Madsen and Nyman, but states explicitly that the monomolecular reaction formula is applicable to the process, "one reagent being represented by the disinfectant" which being present in excess may be regarded as having a constant concentration, "and the second by the protoplasm of the bacterium;" she amplifies this statement by making the supposition that the bacteria undergo rapid cyclic variations in their ability to react with the disinfectant. Phelps[7] in developing Miss Chick's method for standardizing disinfectants, adopts the same explanation. Arrhenius[16] says, "there is no doubt that the different cells in a sample of bacteria or red blood-corpuscles possess a different power of resistance to deleterious substances," but that "the different lifetime of the different bacteria does not, therefore, depend in a sensible degree on their different ability to resist the destructive action of the poison," and accepts Chick's explanation, as does Eijkman,[8] at least in the case of certain bacteria.

The acceptance of such an explanation makes it necessary to assume that loss of viability, like the breaking up of a single molecule of saccharose during inversion, takes place in a single step; in other words, that the disinfectant cannot have any cumulative effect on the viability of individual cells. If the loss of viability occurred in two or more steps, some or all of the cells surviving at any time during the process would be "partially dead," and a greater proportion of them would succumb in any given interval of time than would have done so during the same interval at the beginning of the process when all of the cells were entirely unaffected. In other words the per cent death would increase during the process instead of remaining constant as demanded by the law of monomolecular reactions.

This assumption that death occurs at a bound, as it were, is surprisingly at variance with the usual conception of vital processes. It seems to necessitate that we regard a living cell as being dynamically

comparable to a molecule. For this reason Robertson[17] has offered another explanation for the apparent exponential decrease in the number of surviving cells; *i.e.*, for the apparent applicability of the law of monomolecular reactions. His explanation assumes the collisions with the disinfectant molecules to be distributed fortuitously among the different individuals of a homogeneous group of cells. Since it would be out of place to consider here the details of his mathematical proof, it must suffice to point out that Robertson's quantity x, "the number of units of the underlying change," must apparently be at one and the same time a constant, and an exponential function of time. This impossible assumption is the basis of Robertson's whole proof. Other mathematical inconsistencies occur, but are of relatively little consequence.

In a subsequent paper[6] Miss Chick modifies her original theory by assuming that it is the protein molecules of the bacterial protoplasm, which, like the sugar molecules during hydrolysis, undergo the cyclic changes in energy content, upon which depend their ability to react. The concentration of these protein molecules at any moment would then, according to Miss Chick, determine the rate of death of the bacteria at that moment. We have already seen that under these conditions the cells of a group of uniformly resistant individuals, such as Miss Chick postulates, would all die at one time. If the course of disinfection is to parallel that of the reaction, the diametrically opposite condition must prevail; namely, one in which the cells are equally distributed among all the possible classes of resistance; in other words, the equation of the variation curve must be $y = y_0$.

Von Liebermann and von Fenyvessy[12] explained the hemolytic "monomolecular curve" on the basis of the probable rate of exit of hemoglobin from the individual cell, from which the pigment might be supposed to diffuse at a rate proportional to the difference between the intra- and extracellular hemoglobin concentrations. This explanation is evidently in conflict with the observed progressive decrease in the number of intact erythrocytes, and with the fact reported by Dienes[18] that there is a nearly constant ratio between the hemoglobin

[17] Robertson, T. B., *J. Hyg.*, 1914, xiv, 143.

[18] Dienes, L., *Biochem. Z.*, 1911, xxxiii, 268.

content and the dry weight of the residual cells at various stages of hemolysis.

Since none of the explanations given above is satisfactory let us see whether the experimental facts necessitate the assumption that the equation of the monomolecular reaction is a complete description of the process of disinfection. Examination shows that none of the experiments already referred to include the observations necessary for the study of the first part of the process; the second observation was almost without exception deferred until the process was nearly half complete. It also appears that in many experiments the "initial" number of cells was determined after an appreciable part of the process had occurred, that is after the most fragile cells had already succumbed, and while the reaction was proceeding so rapidly that the slightest errors in the time of sampling would produce great differences in the number of cells recorded.

It is quite impossible to judge correctly the validity of formulas based on such inadequate data. In Fig. 5, I have plotted the points of that one of Chick's curves which shows the best agreement with a monomolecular reaction curve, a similar curve for the number of *Chlamydomonas* cells immobilized at intervals after exposure to dilute hydrochloric acid (Harvey,[15]), a curve for normal serum hemolysis (Henri,[11]), and an original curve (from which the first few observations are omitted) for hemolysis after ultra-violet radiation. In the last two cases hemolysis was estimated colorimetrically. A monomolecular reaction curve is drawn for comparison with these points, and the scales of the abscissæ are so arranged as to make the curves coincide at the point where the effect is half completed. The agreement of the points with the monomolecular reaction curve is about equally close. But in this same figure the curve for the original hemolytic experiment is also drawn with the abscissæ on a larger scale and with the first few observations included. It is obvious that these first few observations are most important. One suspects that had these observers but made sufficient observations during the first part of the process, they would have found that disinfection, killing of seeds, etc., are processes which, like hemolysis, are initially slow and only subsequently attain their maximum velocity. As a matter of fact this is what a number of other workers have found.

Dreyer and his coworkers, for example, found that only after a considerable period of slow laking, did the course of the hemolysis induced by α and β rays of radium and by ultra-violet radiation follow the course of a monomolecular reaction; this initial lag they call, tentatively, a period of induction.

Eijkman[8] has shown that the shape of the disinfection time curve is peculiar to the particular culture of bacteria used, and to some

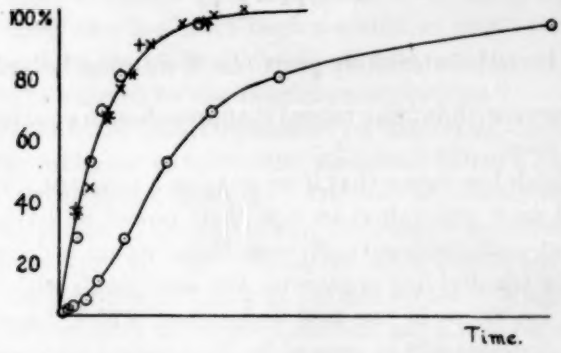

FIG. 5. The upper curve is the monomolecular reaction isotherm, beside which are plotted experimentally determined points for normal serum hemolysis (Henri, asterisks), the immobilization of *Chlamydomonas* by 0.009 per cent HCl (Harvey, diagonal crosses), disinfection (Chick, erect crosses), and hemolysis by ultra-violet radiation (original, circles); the abscissæ were so adjusted in plotting these points as to make their curves coincide with the monomolecular reaction isotherm at 50 per cent. The lower curve represents the course of the same ultra-violet hemolysis, but with the abscissæ plotted on a larger scale, and with the addition of 5 points determined during the first part of the process. The ordinates represent degree of completion of the process, and abscissæ, time.

extent characteristic of the species. Both Eijkman and Reichenbach[9] have secured disinfection time curves which, even when determinations during the early stages of the process are included, do not diverge very greatly from the exponential or monomolecular type of curve. It is possible to explain such curves either by assuming a monomolecular fundamental reaction and a variation curve of the form $y = y_0$, or by assuming a fundamental reaction proceeding at a uniform rate, and a variation curve of the form

$$y = y_0 e^{-kx} \qquad (2)$$

where k is a constant. The latter equation is that of the monomolecular reaction isotherm; but it gives a curve having unlimited range, a condition which makes it undesirable in the case of a limited population; it is better to use a curve of very similar appearance which results if we put $x_1 = 0$ in equation (1) above. We thus introduce the necessary factor of limited range given by x_2. The equation, so modified, becomes

$$y = y_0 \left(1 - \frac{x}{x_2}\right)^{kx_2}$$

while if $\frac{x}{x_2}$ is made indefinitely great (*i.e.* if we consider only the first part of a curve with infinite range) it approaches an equation identical with (2) above.

Reichenbach has shown that if we imagine a constant fraction of the bacteria of each generation to lose their power to divide, and suppose that at each successive division there occurs a decrease in the resistance of the dividing organisms, the individuals with the highest resistance, *i.e.* those of the first generation, which have not since undergone division, will be present in the smallest numbers, and that those of succeeding generations (and which have therefore less resistance), will be present in numbers increasing in geometrical proportion. Under certain conditions such a culture would have nearly the type of variation curve necessary to give the "monomolecular" curve of killing; and Reichenbach finds in individual variation a complete explanation of the observed curves of disinfection. This hypothesis is not, however, applicable to the process of hemolysis, and can therefore have no general significance. The agreement of the middle portion of hemolytic and other similar time curves with the monomolecular reaction curve is probably only a coincidence, and is not of fundamental significance.

Yule[19] has shown that it is possible, theoretically at least, to account for a time curve of hemolysis such as that which I have observed, which, rather than a monomolecular curve, is probably characteristic of the other processes here discussed, even if all the cells are assumed to be equally resistant. This involves the assumption that a certain small number, r, of independent changes suffices to cause lysis. Probably in the case of hemolysis r would not greatly exceed 2. If $r = 1$,

[19] Yule, G. U., *J. Roy. Statist. Soc.*, 1910, lxxiii, 26.

Yule's formulas lead to a "monomolecular" curve. It seems more rational to adopt some explanation which takes into account the individual variations in resistance, thus avoiding unnatural assumptions as to the nature of the fundamental reaction.

IV.

The idea that the rate of disinfection is due to variations in resistance is not new; Geppert[20] gave expression to it nearly a generation ago. The same idea has since been restated in many forms and in many connections, but always without reference to any influence which might be exerted by the fundamental reaction.

Mioni [21,22] criticizes Henri's studies[11] on hemolysis, because Henri neglected the influence of individual variation; Mioni's experimental evidence is inconclusive, however. Dienes[18] suggested that various degrees of resistance to hemolysis were distributed in accordance with "Quetelet's law" ($y = y_0 e^{-kx}$). Hewlett[23] says, with reference to the hypothesis offered by Miss Chick in her first paper, "While admitting that the disinfection of anthrax spores follows the *course* of a unimolecular reaction, I think it extremely doubtful, to say the least, if the reaction between disinfectant and bacterium *is* a unimolecular reaction." Hewlett's own experiments on the effect of mercuric chloride on the viability of mustard seeds accord with those of Darwin and Blackman,[16] but he says: "It appears to me that only by a wide stretch of the imagination can the interaction of mustard seed and disinfectant be considered as a unimolecular reaction, or a reaction of a higher order, yet it follows approximately the course of the former."

Loeb and Northrop[24] also criticize the idea that disinfection is due to a monomolecular reaction. They say Miss Chick "was probably led to such an assumption by the fact that the ascending branch of the mortality curve in her experiments was generally very steep . . . . almost a vertical line, thus escaping detection. Hence she noticed usually only the less steep descending

[20] Geppert, J., *Berl. klin. Woch.*, 1889, xxvi, 789.
[21] Mioni, G., *Ann. Inst. Pasteur*, 1905, xix, 84.
[22] Mioni, *Compt. rend. Soc. biol.*, 1905, lvii, 485.
[23] Hewlett, R. T., *Lancet*, 1909, i, 889.
[24] Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 103.

branch which could be interpreted as a monomolecular curve for the reason that her experiments lasted only a short time." Miss Chick's published curves were, as a matter of fact, time curves, *i.e.* "integrals" of "mortality" or variation curves, and had she been able to make sufficient observations at the beginning of the process, she might have obtained curves at first horizontal, but very quickly turning downwards. Such a condition would find expression in a mortality curve as an ascending branch rising very steeply from zero to a maximum.

The true explanation of the course of processes like disinfection is undoubtedly a combination of the two extreme views: one attributing the course to variation alone, the other considering it to express the nature of the fundamental reaction alone. Both of these factors exert an extremely important influence. We have seen that it is theoretically possible to relegate variation to a position of unimportance by assuming a variation curve having the form $y = y_0$. Such a distribution of various degrees of resistance is obviously unnatural, and the assumption that it occurs must, in the absence of definite evidence, be considered unwarranted. Its use frequently necessitates the postulation of "latent" or "induction" periods of whose existence we have no further proof. On the other hand we may consider the reaction velocity as constant, and by graphic differentiation obtain not unnatural frequency curves. Perhaps by employing several constants we may even obtain applicable equations; but the constants will have no physical meaning, and the equations no general significance.

The reaction velocity, as a moment's consideration will show, must ordinarily decrease as the process goes on; for "sub-minimal" amounts of the toxic substances carry the process to partial completion, and whatever the final "equilibrium" may be, it is gradually attained; the process does not abruptly cease.[25]

[25] Curves drawn through points expressing the amount of toxic agent required to produce various degrees of completion of such processes might be regarded as "integrals" of the frequency curves of resistance. In practice, however, this reasoning is applicable only to such *in vivo* experiments as determination of therapeutic efficiency or toxicity of drugs, radiation, etc. *In vitro*, factors such as bacterial contamination, autolysis, cell division, or starvation are likely to supervene, and distort the observed curve.

Should we not then conclude that the course of processes like disinfection is, like *b* in Fig. 4, the result of the simultaneous operation of two factors: the frequency curve of variation in individual resistance, which may be different for each group of cells and each toxic agent; and the course of the fundamental reaction, which usually proceeds with a velocity diminishing during the experiment at a rate dependent on the particular conditions prevailing? We must also bear in mind that what we have supposed to be the fundamental reaction may be the end result of a complex series of interrelated or "catenary" reactions. If some one link in this chain of events is a change which, from the beginning to the end of the process, is so slow as to govern the rate of the whole series, which taken together is regarded as the fundamental reaction, then, and only then, will orderly laws describe the course of the latter.

This conception does indeed render a solution of the problem much more difficult than it once seemed, but not necessarily unattainable. A proper understanding of this "group experiment," as we might perhaps call the widely employed type of which disinfection is but one example, should lead to better interpretation of the phenomena themselves, and a far deeper insight into the fundamental life processes to which they are due.

CONCLUSIONS.

1. The course of such processes as hemolysis is very largely dependent upon variations in resistance among the different individuals, and secondarily upon the course of the fundamental reaction.

2. The fundamental reaction may be either a simple process, or the expression of a complex series of changes whose rate is at all times governed by that of the slowest of the series. This might perhaps be regarded as another expression of the so called "Law of the minimum."

3. Unnatural assumptions would be requisite for the explanation of a resemblance between the course of such processes in general and that of a monomolecular reaction.

4. The supposition that such a general resemblance exists is not supported by the available evidence.

5. The independent determination of either the nature of the fundamental reaction, or the type of the variation curve for the particular case under observation, will further our knowledge of the nature of such processes and lead to a far deeper insight into the nature and reactions of living matter.

THE LAW CONTROLLING THE QUANTITY OF REGENERATION IN THE STEM OF BRYOPHYLLUM CALYCINUM.

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(Received for publication, June 24, 1918.)

I.

It is well known that isolated pieces of plants and lower animals are able to replace the lost parts by a new growth which is called regeneration. The investigation of this phenomenon by merely qualitative methods of experimentation has led only to verbalisms. Thus it has been stated by Noll that the plant or animal possesses a "morphestesia;" *i.e.*, a consciousness of what its proper form should be and hence the regeneration of lost organs. Driesch applies a similar verbalism calling the morphestesia "entelechy." Weismann suggests that without the power of regeneration many species might have died out—hence regeneration. Others speak of regeneration as the effect of the "stimulus" of the wound. If we wish to substitute for these or similar expressions, which have led and can lead to no scientific result, a theory in the sense in which this word is used by the physicist, we must investigate the problem of regeneration by the methods of quantitative experimentation.

The writer has introduced such a method in the study of regeneration of the plant *Bryophyllum calycinum*, which is unusually favorable for work of this kind, and he has already reported some of the results obtained. The most significant fact was found in the regeneration of the leaf of this plant. When the leaf of *Bryophyllum* is detached from the plant, it will form roots and shoots in its notches. Each node of the stem has two leaves in opposite position—sister leaves —which under normal conditions have equal size. Since they have also the same age and the same history, they possess not only the same mass but contain also very probably chloroplasts in the same number and in the same degree of efficiency; so we can say such sister leaves

have approximately equal masses of *active* substance. The writer was able to show *that such sister leaves of equal weight produce equal masses of shoots in equal times and under equal conditions of illumination, temperature, and moisture.* He found, moreover, that if we reduce the mass of one set of sister leaves by cutting out pieces from the center of the leaves, while the other set remains intact, both sets produce shoots approximately in proportion to their masses, even if the number of shoots produced by the two sets differs widely. We, therefore, can say that *equal masses of sister leaves produce equal masses of shoots in equal times and under equal conditions, regardless of the number of shoots produced.*[1]

This law shows that the problem of regeneration is part of the problem of growth and that it falls under the law of chemical mass action.

Inasmuch as age and previous history influence the active mass of photosynthetic material of the leaf, it is obvious that the law of the production of equal masses of shoots by equal masses of leaves is fulfilled more accurately by comparing sister leaves than it would be by comparing leaves with a different history; *i.e.*, leaves which differ in the mass of photosynthetic material in the unit mass of leaf. In such cases we should have to use statistical methods; *i.e.*, we should be compelled to use much larger numbers of leaves in order to eliminate the influence of variation in the relative mass of photosynthetic and other material in the leaf which is required for the regeneration (growth) of the shoots.

II.

Regeneration in an isolated piece of stem is much more general than regeneration in an isolated leaf, and the question arises whether a similar mass law, as that found for regeneration in a leaf, controls the quantity of regeneration in a stem.

In the axil of each leaf of the stem of *Bryophyllum* is found a dormant bud capable of growing into a shoot (3, Fig. 1). Each node contains two such dormant buds on opposite sides, one in the axil of each leaf. In successive nodes the lines connecting the two leaves

[1] Loeb, J., *Science*, 1917, xlv, 436; *Bot. Gaz.*, 1918, lxv, 150; *Ann. Inst. Pasteur*, 1918, xxxii, 1.

and their axillary buds are at right angles to each other. If we, therefore, split a stem longitudinally into two halves (Fig. 1) in such a way that the plane of section is at right angles to the line connecting the two most apical buds (1, Fig. 1), the section cuts through the buds in the next node (2, Fig. 1), and injures them more or less, while it leaves the buds of the second next (3, Fig. 1) node intact, and so on. It happens for reasons which need not be discussed here that in such a stem as a rule only the most apical bud (1, Fig. 1) grows out.

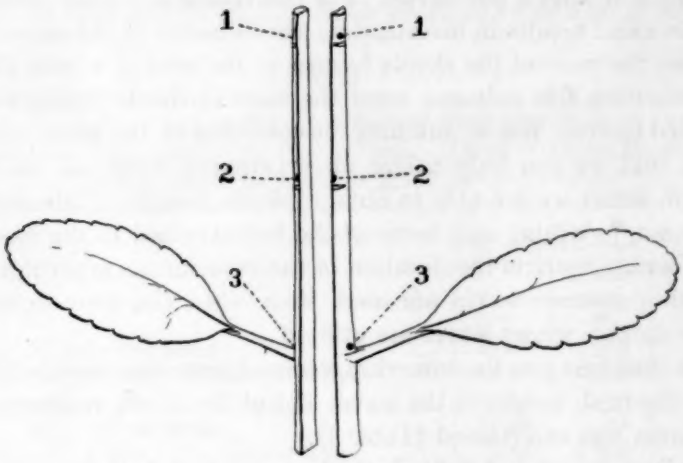

Fig. 1. Diagram showing the method of splitting the stem longitudinally in order to investigate the influence of mass of basal leaf upon shoot production. 1, 2, 3, designate the three pairs of dormant buds, two in each of the three nodes of the stem.

The following method was used for testing whether or not the quantity of regeneration in a piece of stem is controlled by the simple mass law which holds for the regeneration of shoots in the stem. From the stem of *Bryophyllum* were cut pieces containing three nodes (Fig. 1). All the leaves were removed except the two at the basal node. Then the stem was split lengthwise so that each half of the stem contained one basal leaf and one intact bud at the apex (1, Fig. 1), while the two buds (2, Fig. 1), in the middle node were generally injured in the operation. As stated, in most cases only the apical bud grows out in such a piece of stem. By leaving the leaf in one of

the half stems intact, while the sister leaf at the base of the other half stem is reduced in size, it is possible to find out whether the mass of the shoots regenerated at the apex of each half stem bears any relation to the mass of the two leaves. Such experiments were made by us and as a rule six or more different stems were used in one experiment.[2]

Many experiments are lost for the reason that the leaf at the base of a stem wilts or perishes much more rapidly than a leaf entirely detached from a stem; the latter may last for months while the former will usually wilt after a few weeks. For this reason it is more difficult to obtain exact results in investigating the influence of the mass of the leaf on the mass of the shoots formed at the apex of a stem than in investigating this influence upon the mass of shoots regenerated by isolated leaves. But in watching the condition of the leaves and realizing that we can only utilize an experiment when all the leaves remain intact we are able to obtain reliable results. This source of error due to wilting and decay of the leaf attached to the base of a stem which restricts the duration of the experiments is greater when the thin summer leaves are used than when the more fleshy and more durable winter leaves are utilized.

We shall first give the numerical results of some experiments in which only the fresh weight of the leaves and of the shoots regenerated by the stem was ascertained (Table I).

In Experiment 3 of Table I, the leaves of both half stems were left intact; 1 gm. of leaf in one set caused the production of 213 mg. of shoots in the stem, while 1 gm. of leaf of the other set produced 240 mg. of shoots in the apex of the stem. This shows the degree of accuracy to be expected in these experiments.

In the three other experiments the masses of the two sets of sister leaves varied considerably. Thus in Experiment 1 the masses of the leaves were approximately in the ratio of 1 : 6 (2.8 : 19.0); the masses of shoots produced by the two sets of stems were also approximately in the ratio of 1 : 6, namely 0.44 : 2.8. Experiment 2 gave similarly good results. The ratio of the two masses of sister leaves was approximately 1 : 5, namely 3.5 : 18.5, and the masses of shoots produced by the stem were also approximately in the ratio of 1 : 5, namely 0.7 : 3.6.

[2] Loeb, *Proc. Nat. Acad. Sc.*, 1918, iv, 117.

In Experiment 4 the two masses of sister leaves were in the ratio of almost 1 : 5, namely 2.7 : 11.9, and the masses of shoots produced by the stem were also approximately in the ratio of 1 : 5, namely 0.53 : 2.7.

Our experiments have shown without exception that the greater the mass of a basal leaf the greater the mass of shoot regenerated by the apex of the stem in equal times and under equal conditions; and wherever we were certain that the leaves remained normal during the experiment it was also possible to show that the mass of shoots produced by the apex of the stem

TABLE I.

Influence of Mass of Leaf at Base of a Piece of Stem upon Mass of Shoot Regenerated at the Apex of the Piece of Stem. Apices of Leaves Dipping in Water, Stems Suspended in Moist Air (See Fig. 2).

No. of experiment.	Duration of experiment.		Fresh weight of leaves.	Fresh weight of 6 regenerated shoots on stem.	Regenerated shoots per gm. of leaf.
	days		*gm.*	*gm.*	*mg.*
1	37	6 whole leaves	19.030	2.808	146
		6 sister " reduced in size	2.853	0.443	152
2	34	6 whole leaves	18.490	3.586	194
		6 sister " reduced in size	3.503	0.668	186
3	26	6 whole leaves	7.128	1.511	213
		6 " sister leaves	8.142	1.963	240
4	33	6 whole leaves	11.878	2.728	229
		6 sister " reduced in size	2.740	0.530	200

varied approximately in direct proportion to the mass of the leaf attached to the base of the piece of stem.

Fig. 2 is a drawing of the appearance of a group of six pairs of half stems having a leaf at the base. The duration of the experiment was 21 days. The upper half stem with a whole leaf is always the sister piece of the half stem with a reduced leaf immediately below it. It is obvious that the stems with the reduced leaves have smaller shoots than those with whole leaves. The apices of the leaves were dipped in water, the stems were in moist air. Fig. 3 is a photographic

reproduction of the same experiment (with left and right sides reversed).

These experiments prove that *equal masses of leaves at the base of a piece of stem cause the production of approximately equal masses of shoots at the apex of the stem in equal times and under equal conditions.*

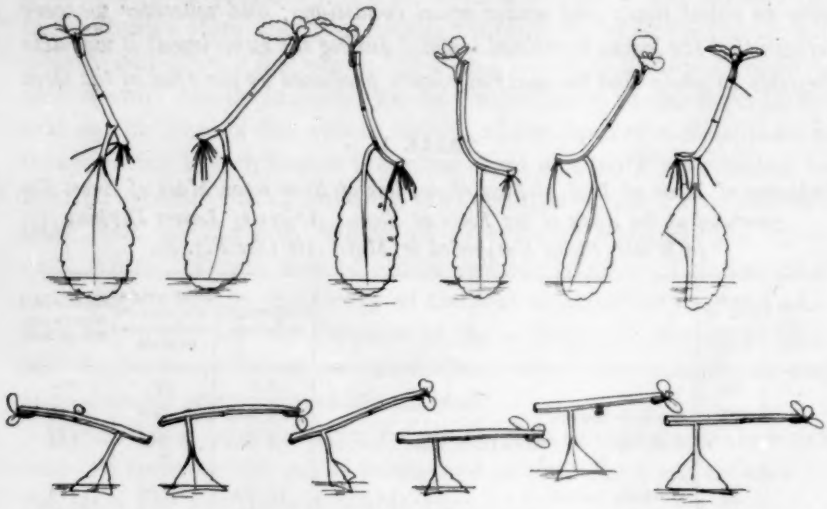

Fig. 2. Drawn after nature. The upper specimen with whole leaf and the one beneath it with reduced leaf are always the two halves of the same piece of stem split lengthwise in the way indicated in Fig. 1. The shoots at the apex of the stem with whole leaves (upper row) are considerably larger than the corresponding shoots with the sister leaf reduced in size (lower row). As a matter of fact, the masses of regenerated shoots were in proportion to the masses of the leaves attached to the base of the stems. The reader will also notice that the stems with whole leaves (upper row) have formed roots at their base, while those with reduced leaves have not yet formed any roots (though they did so later). It is also noticeable that the shoots with whole leaves show geotropic curvature, while those with reduced leaves are still straight.

Even where some of the leaves were wilted the mass of shoots produced at the apex was always greater when the mass of leaves was greater, but it was no longer possible to prove approximate proportionality though such proportionality in all probability existed.

In the experiments presented in Table II the stems as well as the leaves were suspended in moist air to eliminate the influence of ab-

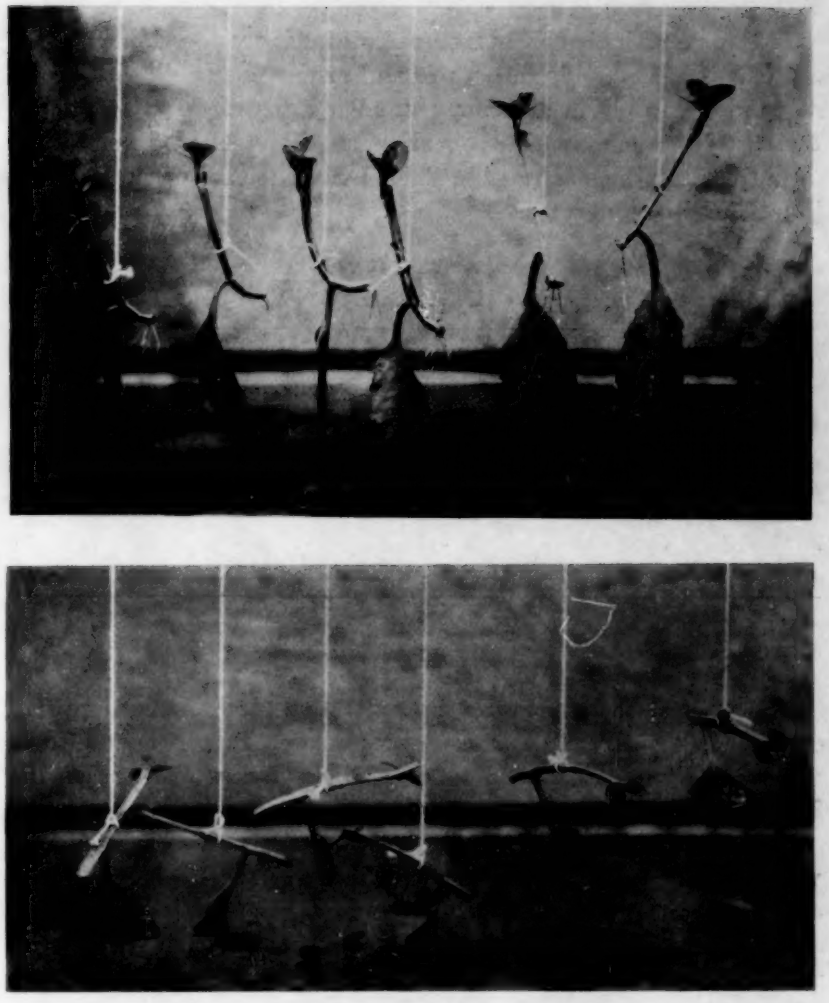

Fig. 3. Photograph showing the same experiment as in Fig. 2. (Left and right are reversed in Figs. 2 and 3.)

sorption of water by the leaf. The duration of these experiments had to be short on account of the more rapid wilting of the leaves connected with the stems.

In this case the disturbing factor of wilting is greater than when the apices of the leaves dip into water and hence the results with leaves suspended entirely in air show a smaller approximation to proportionality between mass of leaves and mass of shoots produced by the stem than that demonstrated in Table I, although the proportionality is not entirely obliterated.

TABLE II.

Stems and Leaves Suspended in Moist Air.

No. of experiment.	Duration of experiment.		Fresh weight of leaves.	Fresh weight of 6 regenerated shoots on stem.	Regenerated shoots per gm. of leaf.
	days		*gm.*	*gm.*	*mg.*
1	17	6 whole leaves	4.376	0.524	119
		6 " sister leaves	3.264	0.494	151
2	19	6 whole leaves	6.662	1.126	168
		6 sister " reduced in size	2.853	0.6885	237

III.

The mass of shoot produced in an isolated piece of stem entirely deprived of leaf is small compared with that produced when a leaf is attached to the base of the stem. This was demonstrated in the following experiment.

Six stems, each containing three nodes and one leaf at the base, were split longitudinally in the way described above. One half stem contained one leaf at the base, the other had no leaf. To insure an equal water supply to the stems they were put with their cut sides on moist filter paper (Fig. 4), the leaf at the base of the one set of stems being in moist air. The duration of the experiment was 24 days.

Fresh weight of shoots produced in 6 half stems *without leaves*, 0.120 gm. (dry weight, 0.0105 gm.).

Fresh weight of shoots produced in 6 half stems *with leaves*, 2.088 gm. (dry weight, 0.2015 gm.).

Weight of 6 leaves, 8.262 gm.

The dry weight of the 6 stems without leaves was 0.286 gm. and the fresh weight was 3.935 gm. The dry weight of the 6 half stems containing a leaf was 0.688 gm. and the fresh weight 6.203 gm. We have already shown in a previous paper that the mass of an isolated piece of stem increases when a leaf is attached to it.[3]

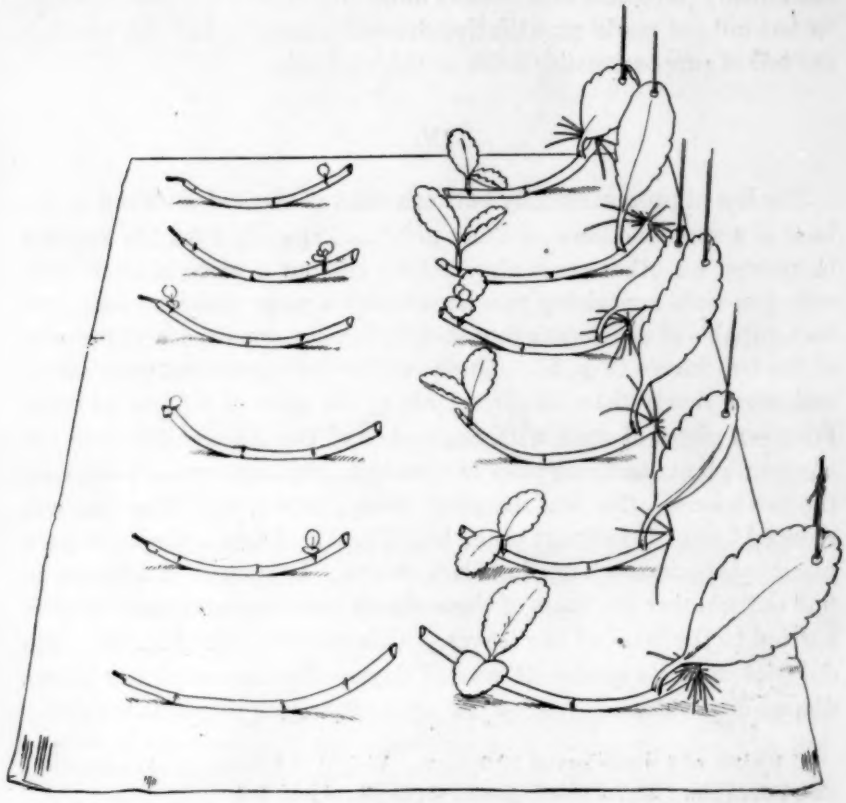

Fig. 4. Showing the enormous difference in mass of shoots regenerated by a half stem with leaf at base and the other half stem without leaf. The stems rested with their cut surface on moist filter paper.

Hence we can say that only about 5 per cent of the material of the regenerated shoots was contributed by the stem and about 95 per cent of the mass of the regenerated shoots of the stem was formed from

[3] Loeb, *Ann. Inst. Pasteur*, 1918, xxxii, 1.

material furnished by the leaf. This is not surprising since we know that the leaf is the organ where the material for new growth in the plant is manufactured. It is also to be expected that in pieces of stems of different mass but without leaves the mass of shoots regenerated will increase with the mass of the piece of stem. The writer has already published experiments indicating that this is true,[4] though he has not yet made quantitative determinations to find out whether the law of proportionality holds in this case also.

IV.

The law of proportionality between mass of the leaf attached to the base of a stem and mass of shoot produced apically from the leaf can be proved for other cases also. If we cut out a piece of stem with only one node containing two leaves such a piece possesses only two buds capable of developing into shoots; namely, one in each of the axils of the two leaves (Fig. 5). These axillary buds grow out more rarely and more slowly than the free buds at the apex of a piece of stem. Fourteen pieces of stem with one node and two leaves each were cut out from plants and each piece of stem was split longitudinally between the two leaves. One leaf remained always intact, the other leaf was reduced by cutting off part of the leaf (Fig. 5). Eight of these fourteen pieces of specimens formed axillary shoots. It seemed of interest to find out whether the mass of these shoots was approximately in proportion to the mass of the leaves. This was the case (Fig. 5). The duration of the experiment was 45 days. The apices of the leaves dipped into water.

a. Weight of 8 whole leaves, 10.968 gm. Weight of 8 shoots produced in their axil, 1.8025 gm. Mg. of shoots produced per gm. of leaf, 164.

b. Weight of 8 reduced sister leaves, 3.586 gm. Weight of 8 shoots produced in axil, 0.5895 gm. Mg. of shoots produced per gm. of leaf, 164.

The mass of axillary shoots produced by each set of sister leaves was, therefore, in direct proportion to the mass of the leaves.

[4] Loeb, *Bot. Gaz.*, 1915, lx, 249.

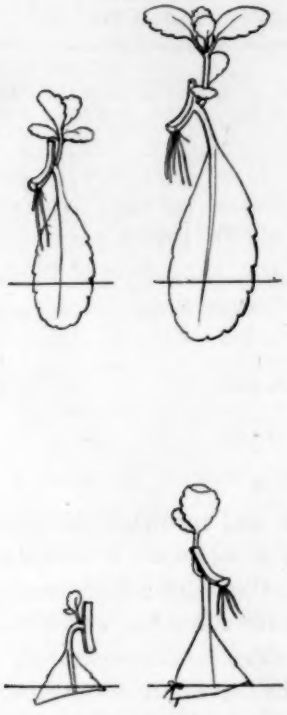

Fig. 5. Showing influence of mass of leaf on mass of shoot produced in the axil of each leaf. Each upper specimen with whole leaf and the specimen below it with reduced leaf are halves of the same stem.

V.

Since it might be argued that the leaves furnish only water for the growth of the shoots, it was necessary to find out whether the dry weight of the shoots regenerated by the stem varies also with the dry weight of the leaves attached to the base of the stem. The dry weight of the basal leaves and of the shoots regenerated by the stem was ascertained by drying these organs in an electric oven over night at 100°C. Some of the experiments mentioned in Tables I and II were used for this purpose and in Table III the experiments in which the dry weights were determined are indicated.

The leaves, therefore, do not only supply water for the regeneration of the shoots by the apex but dry matter as well. This conclusion is supported by the experiments to be discussed in the next chapter.

TABLE III.

No. of experiment.		Dry weight of leaves.	Dry weight of shoots.	Dry weight of shoots per gm. of dry weight of leaf.
		gm.	*gm.*	*mg.*
4, Table I.	6 whole leaves	0.440	0.2135	0.485
	6 sister " reduced in size	0.1055	0.0430	0.407
2, Table II.	6 whole leaves	0.3700	0.1002	0.271
	6 sister " reduced in size	0.1991	0.0632	0.317
1, Table II.	6 whole leaves	0.3144	0.0486	0.154
	6 " sister leaves	0.2585	0.0450	0.177

VI.

If it is true that the leaf furnishes the material to the stem from which the regenerating shoot grows, it should be possible to show that the basal leaf in connection with a regenerating stem has (after some time) less weight than the sister leaf which is separated entirely from the stem. This can indeed be demonstrated. A piece of stem is cut from a plant and all the leaves are removed except the two leaves at the basal node of the stem. One basal leaf is entirely separated from the stem to serve as a control; the other leaf remains in connection with the stem.

Six stems, about 6 cm. long, with several nodes and with a basal pair of leaves of approximately or practically the same size were selected for the experiment. Such sister leaves of the same size have also practically the same mass as shown in previous experiments. Both the isolated leaves and the stems with one leaf attached were suspended in moist air, in the same aquarium under identical conditions of light, temperature, and moisture. After 16 days the fresh and dry weights of the two sets of leaves, namely of the detached leaves and of their sister leaves connected with the stem, were ascertained. It was found that the leaves connected with the stems weighed considerably less than the detached leaves, and the difference was far in excess of the natural variation in the weight of fresh sister leaves of equal size.

Experiment I. Leaves and Stems in Moist Air.

	Fresh weight. gm.	Dry weight. gm.
6 detached leaves, at end of experiment	8.242	0.826
6 sister leaves connected with stem, at end of experiment	6.476	0.578

Hence the leaves connected with the base of the stem had given off to the latter about one-third of their dry weight.

During this time the detached leaves formed roots and tiny shoots in some of their notches while nothing of this kind occurred in the leaves connected with the stems. Instead twelve shoots were formed at the apices of the six stems and the twelve shoots weighed fresh 0.528 gm. and dry, 0.068 gm. The inference is that the material for the latter came from the leaves, but apparently more material than this was given off by the leaves to the stems. We have indeed shown in a previous paper that aside from the material for shoot formation the leaf sends material into the stem which may be used for the growth of certain tissues in the stem, resulting in callus formation, geotropic curvature, and increased thickness of the stem.[3]

The experiment was repeated with this difference, that the apices of the leaves dipped into water, while the stem and the rest of the leaves were suspended in moist air. Both sets of leaves were in the same aquarium under equal conditions of temperature, light, and moisture, and both were of equal size and mass at the beginning of the experiment. The experiment lasted 18 days.

Experiment II. Apices of Leaves Dipping in Water; Stems Suspended in Moist Air.

	Fresh weight. gm.	Dry weight. gm.
6 detached leaves, at end of experiment	11.159	0.791
6 sister leaves attached to base of stem, at end of experiment	4.485	0.388

Hence the leaves gave off to the stem about 50 per cent of their dry weight.

This difference is only partially accounted for by shoot production in the stem, the total shoot production amounting to 1.166 gm. fresh and 0.111 gm. dry weight.

Many more experiments than these were made and in some the leaves were weighed both at the beginning and at the end of the

experiment.[5] They leave no doubt of the fact that the basal leaf gives off a considerable amount of its material to the stem.

We have, therefore, a right to conclude that a basal leaf gives off material to the stem, part of which is used for the formation of shoots in the stem.

Experiments were carried on in the dark and it was found that the mass of shoots regenerated under these conditions was small even when a large basal leaf was attached to the stem; and no proportionality between mass of leaf and of shoots regenerated on the stem could be found. This indicates that the products of assimilation in the leaf are part of the material used for the regeneration of the shoots on the stem.

Theoretical Remarks.

These experiments have shown that in the case of the regeneration of the leaf as well as of the stem the quantity of regeneration is determined by the mass of material sent out by the leaf and manufactured in the leaf; and possibly also to a small extent by material manufactured in the stem or circulating or stored in the stem at the time the stem was cut out from the plant.

The same simple mass law seems to hold for the quantity of roots regenerated at the basal end of the stem or basally from a leaf attached to the stem. It was very obvious in all our experiments that the mass of roots formed in a piece of stem in a given time increases with the mass of a leaf and the roots commence to grow out later when the leaf is smaller. The writer has, however, not yet made enough quantitative measurements to permit him to state that there is a strict proportionality between mass of leaf and mass of roots regenerated.

In animals the blood and lymph play the same rôle as does the sap in plants, and we may surmise that the quantity of sugar, amino-acids, salts, and of "accessory substances" in the body fluids determines the quantity of regeneration in animals. In an animal, regeneration may occur even when no food is taken up, and it is to be assumed that the tissues of a fasting organism constantly convert some of the material stored in the cells into sugar, amino-acids, and vitamines,

[5] We will omit these experiments here since they will be discussed in one of the following papers.

which diffuse into the blood and become available for the growth of the regenerating tissues; in other words, it is the constant hydrolysis going on in the organism which supplies the material for the growth and regeneration of fasting animals. It remains for future investigations to find out whether the ratio between mass of growth material circulating in the blood or lymph and the quantity of regeneration obeys also the simple mass law established for regeneration in *Bryophyllum*.

For those interested in the dynamics of these processes attention may be called to the fact that in our experiments the action of two masses of active material, m and m_1, in two sister leaves is compared. These masses are certain constituents of the two sister leaves, primarily the chlorophyll, and their ratio $\frac{m}{m_1}$ may be considered approximately constant throughout the duration of the experiment. Our experiments have furnished the proof that the ratio of the mass of shoot regeneration in two halves of a stem (each possessing a leaf at the base) is approximately proportional to $\frac{m}{m_1}$; or in other words, that the law controlling the quantity of shoot regeneration of the stem is a special case of the law of mass action.

SUMMARY.

1. A method is given which allows us to measure the influence of the mass of a leaf upon the quantity of shoots regenerated in an isolated piece of stem. This method consists in isolating a piece of stem with only two leaves left at the basal node and then splitting the stem lengthwise so that each half has one basal leaf. By leaving one leaf intact while the size of the sister leaf is reduced, the influence of the mass of the leaf upon the quantity of shoots regenerated by the stem can be measured.

2. This method has yielded the result that the mass of shoots regenerated at the apex of such a piece of stem increases under equal conditions and in equal time with the mass of the leaf, and is approximately proportional to the mass of the leaf.

3. Such an influence of the mass of the leaf upon the mass of shoots produced by the stem is only intelligible on the assumption that the

growth of the regenerating shoot occurs at the expense of material furnished by the basal leaf.

4. This assumption is supported by two facts: first, that in the dark this influence of the leaf disappears more or less completely; and, second, that a leaf attached to the base of a regenerating stem after some time weighs markedly less than does a sister leaf completely detached from the stem, but otherwise under equal conditions.

5. This latter fact that a leaf when attached to the base of an excised piece of stem wilts more rapidly than when completely isolated is the reason that the proportionality between mass of a basal leaf and mass of shoot regenerated at the apex of an isolated piece of stem cannot always be demonstrated with the same degree of accuracy as the proportionality between the mass of completely isolated leaves and the mass of shoots they produce.

6. The material furnished by the leaf to the stem is not restricted to water but includes also the solutes, since not only the fresh weight but also the dry weight of the shoot regenerated by a piece of stem increases with the mass of the leaf attached to the base of the stem; and since not only the water contents but the dry weight of a leaf attached to the base of an excised piece of stem diminish when compared with the dry weight of a completely detached sister leaf.

7. The mass of shoots produced by an isolated piece of stem without leaf is small and almost negligible compared with the mass of shoots produced by the same piece of stem when a leaf of sufficient mass is attached to the base of the stem.

REVERSAL OF REACTION BY MEANS OF STRYCHNINE IN PLANARIANS AND STARFISH.

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(Received for publication, June 20, 1918.)

Experiments on the Planarian, Bdelloura.

It has been shown that a single excitation at a median point in the earthworm elicits a shortening of the body anterior and a lengthening posterior to that point. Active extension of the body is produced by contraction of the circular muscles, and by inhibition of contraction of the longitudinal muscles of the body. This is a case of reciprocal innervation.[1] Further, it has been found that in the earthworm, just as in vertebrates, strychnine converts inhibition into excitation, thus causing upon stimulation a contraction of the longitudinal muscles, which results in a shortening posterior to the point of stimulation.[2]

Flatworms have, in addition to longitudinal and circular systems of muscles, transverse muscle fibers. These, with the circular muscles, cause an increase in length and decrease in width and thickness of the animal upon contraction, while the shortening and thickening characteristic of the quiescent position are necessarily associated with a contraction of the longitudinal musculature and a relaxation of their antagonists. This implies also reciprocal innervation.

In order to determine whether the nervous mechanism in flatworms is similar in its reaction to that of the earthworm, the following experiments were made with specimens of *Bdelloura*. This is a marine flatworm, ectoparasitic on *Limulus*. If an active individual is touched with an instrument such as a pair of forceps, it stops locomotion, shortens and thickens; *i.e.*, the longitudinal muscles contract and the circular and transverse muscles relax. In case the animals are first

[1] Garrey, W. E., and Moore, A. R., *Am. J. Physiol.*, 1915, xxxix, 146.

[2] Knowlton, F. P., and Moore, A. R., *Am. J. Physiol.*, 1917, xliv, 490.

strychninized by placing them for a few minutes in a solution of strychnine sulfate (1 : 10,000), mechanical stimulation produces the opposite effect; *viz.*, extreme extension and activity of the animal. The action of the strychnine is, therefore, to convert the inhibition of the transverse and circular muscles in the normal response into an excitation.

Experiments on the Starfish, Asterias forbesii.

Animals which orient themselves by means of contact sensitivity or stereotropism are in a state of motor equilibrium when their sensitive (ventral) regions are in contact with a surface. When this normal relationship is disturbed, as by putting the animal on its back, rapid and exaggerated body movements take place, until by chance, the specific receptors come into contact with a surface. Immediately from this point of contact excitatory and inhibitory impulses are sent out, the effect of which is to bring the animal without further waste effort into its normal orientation again.

In the starfish the tube feet are the stereosensitive organs. In an inverted specimen of normal vigor all the arms show initial twisting movements. From the arm whose tube feet first get into contact with a solid surface an excitatory impulse starts to an adjacent arm, as from A to B (Fig. 1), causing the latter to twist as so to face A ventrally and to attach its tube feet to the solid surface. Inhibitory impulses passing from A to D and from B to C cause D and C to release any initial hold they may have had and to bring E more or less passively with them, thus turning a somersault over A and B.[3]

This reaction evidently implies reciprocal innervation. If it were possible to reverse the functioning of this system by means of strychnine, we would have a still closer parallel with the corresponding mechanism in vertebrates. The similarity of the reaction of the earthworm to that of the vertebrate in this respect, is perhaps to be expected since the histology of the nervous system of the annelid also shows the synaptic structures. But because the nerve tracts of the starfish do not contain elements histologically similar, we must suppose that the strychnine acts, if it acts at all, on certain chemical elements of the neuron, rather than upon some special anatomical

[3] Moore, A. R., *Biol. Bull.*, 1910, xix, 235; *Am. J. Physiol.*, 1910, xxvii, 207.

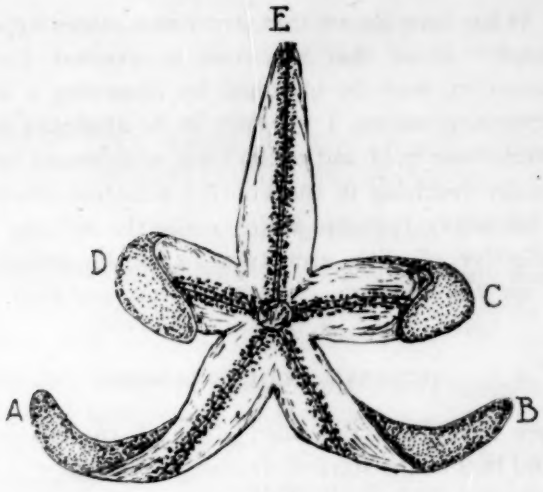

FIG. 1.

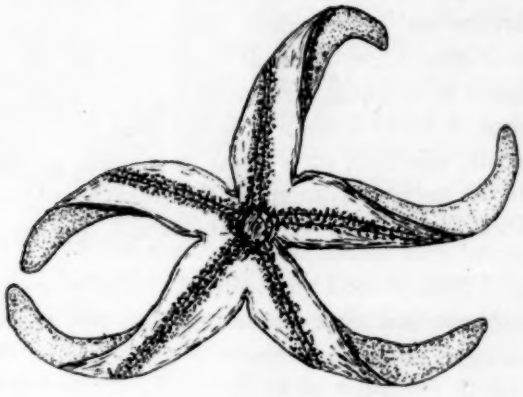

FIG. 2.

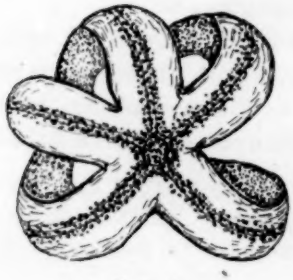

FIG. 3.

structure. It has been shown that strychnine causes hyperirritability in the starfish.[4] Proof that inhibition is reversed, *i.e.*, converted into an excitation, may be obtained by observing a strychninized starfish (strychnine sulfate 1 : 10,000) in its attempts to right itself. All of the arms take hold and retain their attachment to the bottom (Fig. 2), finally resulting in the knotted situation shown in Fig. 3. The usual inhibitory impulses which make the righting possible are no longer effective; all of the arms twist so that the tube feet maintain their hold on the bottom. Strychnine has reversed the normal inhibition.

SUMMARY AND CONCLUSIONS.

Two cases have been described, that of the marine planarian *Bdelloura* and that of the starfish *Asterias forbesii*, in which strychnine reverses reciprocal inhibition. These facts indicate that the nervous systems of these invertebrates function in a manner similar to those of the earthworm and vertebrates. Moreover, it would seem that strychnine acts upon some chemical component of the neuron which is always present in synaptic structures but which also occurs in the simpler neurons of lower forms. The fact that strychnine is without this characteristic effect on such forms as medusa and sea anemone, indicates that the nervous systems of the starfish and planarian have chemical affinities with the vertebrates which the cœlenterates do not possess.

[4] Moore, *J. Pharm. and Exp. Therap.*, 1916, ix, 167; *Proc. Nat. Acad. Sc.*, 1917, iii, 601.

LIGHT AND THE MUSCLE TONUS OF INSECTS. THE HELIOTROPIC MECHANISM.

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(Received for publication, July 23, 1918.)

The profound influence which light exerts upon the activities, movements, and behavior of most insects, bespeaks its deep seated effects upon the physiological processes within the neuromuscular system of these forms. Our investigations have established a direct relationship between the tonus or tension of the skeletal muscles and the illumination of the eyes of heliotropic insects, and show that the coordinated movements of these animals, like those of vertebrates, are dependent upon the maintenance of an adequate and balanced state of tonus in symmetrical groups of muscles.

It was pointed out in a preceding communication on this subject, that in the movements of heliotropic insects, the orientation was determined by the changes in muscle tension induced by unequal illumination of the two eyes.[1] These results were in conformity with the theory of heliotropism proposed by Loeb in 1888,[2] and restated by him as follows: "The mass of photochemical reaction products formed in the retina . . . . influences the central nervous system and through this the tension . . . . of the muscles. If the rate of photochemical reaction is equal in both eyes this effect on the symmetrical muscles is equal; . . . . as a consequence the animal will not be deviated from the direction in which it was moving. This happens when the axis or plane of symmetry of the animal goes through the source of light, provided only one source of light be present. If, however, the light falls sidewise upon the animal, the rate of photo-

[1] Garrey, W. E., *Proc. Nat. Acad. Sc.*, 1917, iii, 602.

[2] Loeb, J., Sitzungsber. physik.-med. Ges., Würzburg, 1888; Der Heliotropismus der Tiere und seine Uebereinstimmung mit dem Heliotropismus der Pflanzen, Würzburg, 1890.

chemical reaction will be unequal in both eyes and . . . . . as a consequence the direction in which the animal moves will change."[3]

The validation of this theory rests upon presenting proof that light acting upon photosensitive organs does affect the tension (tonus) of muscles, and that asymmetrical effects are produced by unequal illumination of the eyes of heliotropic forms. A typical example of the reflex muscle tonus produced by light is noted in the contraction of the sphincter iridis of the vetebrate eye when the retina is illuminated. The degree of contraction is proportional to the intensity of the illumination, it is quite independent of the rate of change in the illumination; furthermore the contraction is a true tonus which is maintained as long as the light affects the retina. Exactly the same type of reaction was described by Loeb for certain sessile heliotropic animals, *Spirographis*, *Eudendrium*, which respond to unilateral illumination by bending toward the light.[4] The muscles contract on the illuminated side and like the sphincter of the iris, remain in tonus as long as the light continues to act. Loeb and Ewald and Loeb and Wasteneys[5] showed that this reaction of *Eudendrium* was proportional to the product of the intensity of the light into the duration of illumination, thus following the photochemical law of Bunsen and Roscoe.

In the case of the motile larvæ of the marine worm, *Arenicola*, which are positively heliotropic, Lillie states that there is an "increase of muscular tone under strong illumination" and that an "inequality of tone on the two sides of the body will result when one side is more strongly illuminated than the other," the muscles of the more strongly illuminated side being in stronger contraction. Thus "heliotropic orientation is a purely muscular phenomenon."[6] Mast[7] showed that this reaction was due to the unequal illumination of the eye spots and Garrey[1] showed that the difference in contractile state (tonus) of the muscles of the two sides of the body of *Arenicola* larvæ persists as long as the difference in the illumination of the eye spots is

[3] Loeb, The organism as a whole, New York and London, 1916, 258.

[4] Loeb, *Arch. ges. Physiol.*, 1890, xlvii, 391.

[5] Loeb, J., and Ewald, W. F., *Zentr. Physiol.*, 1914, xxvii, 1165. Loeb, J., and Wasteneys, H., *J. Exp. Zool.*, 1917, xxii, 187.

[6] Lillie, R., *Am. J. Physiol.*, 1901, v, 59, 60.

[7] Mast, S. O., Light and the behavior of organisms, London, 1911.

maintained and is proportional to the difference in illumination of the two sides. The tonic changes produced under these conditions are entirely analogous to the changes in muscle tonus produced under the influence of a gradually increasing galvanic current and are in marked contrast to the twitch with which a muscle responds to a sudden stimulus. The analogies between electrotonus and what we may now call *phototonus*, have been considered in some detail in a previous communication.[1]

Lyon found[8] that all compensatory motions of insects were abolished by blackening their eyes, while the positions of the eyes of crustacea were so altered that he concluded that "light may cause . . . . an unequal tension of associated muscles." Ewald[9] confirmed this in a quantitative way for the orientation of the eye of ***Daphnia***. Holmes[10] (1908) also describes an "increase in the tension of the leg muscles (and eye stalk of fiddler crabs) brought about by strong illumination." Delage,[11] in 1887, had already concluded that the eyes of insects seem to be their most important organs of equilibrium, and Rádl[12] believed them to be true organs of muscle tonus. Even vertebrates show that illumination of the eyes has a distinct effect upon the tonus of associated muscle groups as was demonstrated by Garrey,[13] and by Lyon's proof that the rheotropic orientation of several species of fish depended upon the optical effects of a "relative motion between the fish and its solid surroundings."[14] Loeb's[15] experiments on the compensatory motions of horned toads show a similar influence of light upon the tonus of associated muscle groups, since the effects of light and rotation, upon compensatory motions, were summed algebraically. In man, the eyes also have an effect upon the tonus of the body muscles, subordinate only to that of the internal ear and the muscle sense. This is illustrated in the so called visual nystagmus of the eyes. It may prove true that the relation of the eyes to Rom-

[8] Lyon, E. P., *Am. J. Physiol.*, 1900, iii, 86.
[9] Ewald, W. F., *Science*, 1913, xxxviii, 236.
[10] Holmes, S. J., *J. Comp. Neurol. and Psychol.*, 1908, xviii, 493.
[11] Delage, Y., *Arch. zool. exp. et gén.*, 1887, series 2, v, 1.
[12] Rádl, E., Untersuchungen über den Phototropismus der Tiere, Leipsic, 1903.
[13] Garrey, *Biol. Bull.*, 1904–05, viii, 79.
[14] Lyon, *Am. J. Physiol.*, 1905, xii, 149.
[15] Loeb, *Arch. ges. Physiol.*, 1907, cxvi, 368.

berg's sign in tabes dorsalis is due simply to a loss of muscle tonus when ocular illumination is prevented rather than to true vision; if so, it falls into the same category with the loss of tonus resulting from the degeneration of the posterior columns whereby the afferent impulses from the muscles and tendons fail.

Ewald, Lee, Lyon, Luciani and others have shown that the muscle tonus of vertebrates is maintained chiefly through afferent impulses from different parts of the otic labyrinth. Ewald states that loss of these impulses, by ablation or defect of the labyrinth, causes "an abnormal relaxation of the affected muscles, diminished energy during activity, and diminished precision of the movements in which they are concerned."[16] The insecta show no lack of muscular tone or power of precision or coordination of movements, although they possess neither otic labyrinth nor structures which suggest that they might function in any similar way. It is the intention of this report to show that the reflex effects produced by illumination of the eye of heliotropic insects establish physiological conditions in their muscles which are in every way analogous to these effects produced through the internal ear in vertebrates. The initial experiments which suggest this analogy were made by Rádl[17] but the observations of Holmes[18] (1905) had a much more direct bearing upon the problem in hand. He worked with the water scorpion, *Ranatra*, which is positively heliotropic; it seems to have been especially favorable material since both the descriptions and drawings of Holmes show striking changes of posture produced by illuminating the animal from different directions, and by blackening the eyes. Our investigations show that similar postures assumed by insects are the result of changes in the tonus of various muscle groups due to the unequal or asymmetrical illumination of the two eyes, a feature which is of general application to a great many groups of the insects.

Our experiments were conducted mainly upon the robber flies which are possessed of long legs and powerful muscles. *Proctacanthus*, (Fig. 1) was especially good material for postural studies, *Promachus*

[16] Ewald, J. R., Physiologische Untersuchungen über das Endorgan des Nervus octavus, Wiesbaden, 1892.

[17] Rádl,[12] p. 52.

[18] Holmes, *J. Comp. Neurol. and Psychol.*, 1905, xv, 305.

and *Deromyia* were also good, but the commoner gray forms of asilidæ did not show the reactions with the same constancy. Other forms will be mentioned when used. To obtain the desired difference in illumination, the eyes were coated wholly or in part by asphalt black varnish which forms a hard, brittle, opaque covering.

Effect of Blackening Both Eyes.—Lyon[19] noticed that house flies with both eyes blinded "show marked loss of equilibrium and often refused to fly or move in a normal manner," if "placed on their backs they would remain there for a long time without making any effort to get up." Similarly we find that *Proctacanthus* remains quiet after

FIG. 1. Robber fly (*Proctacanthus*) under normal conditions, seen from above

blackening both eyes. When handled, the efforts to escape are feeble and unaccompanied by the vicious biting of normal robber flies. When stimulated, they move about in a most incoordinated way and usually topple over on one side or the other. Flight is without lifting power; they fall to the floor and land on their backs. Efforts to right themselves are feeble, incoordinated, and often ineffectual. When resting on a flat surface, the legs are abnormally extended and the proboscis and ventral aspect of the thorax touch the surface, as if the body weight could not be supported. Butterflies similarly settle to the surface, right themselves with difficulty, and simply flutter about, when thrown into the air, and fall to the ground.

[19] Lyon,[8] pp. 111, 112.

In the light, normal *Proctacanthus*, and flies in general, hold the body well from the surface when walking and leave only the tracks of the feet on smoked paper, but after blackening the eyes, or in the dark, some part of the body also leaves a trail. The whole experimental picture of inactivity, muscular weakness, and incoordination, when the eyes are darkened, points to a decreased neuromuscular tonus which is normally maintained reflexly by the action of light on the eyes.[20]

Effects of Blackening One Eye.—The fact that positively heliotropic flies, with one eye removed, move in circles toward the good eye was discovered by Loeb,[2] and Parker[21] found that when one eye of *Vanessa antiopa* was blackened, the animal circled in the same way both when flying and creeping and he pointed out the relation of this phenomenon to the theory of heliotropism. Holmes showed the same behavior of other forms, such as *Hyalella*, *Orchestia*, and *Talorchestia*, and also that the postures of the legs of the two sides of *Ranatra* were different under these conditions.[10,18] Both the circus motions and the postural changes due to unequal illumination of the two eyes, or produced by blackening one eye, are in reality shown, in some degree, by practically all heliotropic insects.

If the right eye of *Proctacanthus* is blackened, the insect circles to the left both when walking and flying. The reason for the abnormal progressive movements is evident if one examines the resting posture of the robber fly, which is more striking in this insect than in any other examined (Fig. 2). On the left side, that of the uncovered eye, the legs are all in a state of flexion, closer together than normally, and drawn well under the body, while on the other side all the legs are extended and spread out like the ribs of an open fan. The anterior leg of the normal side is adducted to the right, *i.e.* to the side of the blackened eye, and may even cross the corresponding leg of that side. The condition is a sustained, tonic one, by virtue of which the only

[20] We offer the suggestion that the restlessness of these insects in the light, and their quietness in the dark, may be a true photokinesis, that in reality both photokinesis and heliotropism have an identical physiological basis in the effects of light which reflexly increases the neuromuscular tonus and excitability, and that it is only the orienting effect of light which prevents the heliotropic forms from exhibiting photokinesis and thus coming to rest in the dark.

[21] Parker, G. H., Mark Anniversary Volume, 1903, 455.

possible movement is one in which the flexed legs pull, and the extended legs push the animal toward the illuminated eye. The legs on the side of the illuminated eye cannot be widely separated nor can those of the other side be easily approximated, thus tending to produce a wider arc of progression on the side of the blackened eye. Coupled with the asymmetrical position of the legs there is a tilting of the whole body toward the side of the unblackened eye, so that the legs on that side may be pressed to the table (Fig. 2). That the body muscles are also involved in this tilting is probable, for in some individuals, especially with strong illumination, the muscles of the

FIG. 2.

FIG. 3.

FIG. 2. Robber fly with right eye blackened, seen from above as in Fig. 1. Body tilts to the left, head rotated still farther to left. Left legs flexed, right legs extended so that, in moving, the fly circles to the left only.

FIG. 3. Robber fly with right eye blackened, viewed from the front. Posture as in Fig. 2.

abdominal segments are contracted on the side of the unblackened eye, thus producing a slight bending of the abdomen with its concavity toward that side. The head is also twisted, with the crown to the left, farther than can be accounted for by the tilting of the body (Fig. 3). This means that the head is actually rotated on the long axis of the body; at the same time the head is also turned with the blackened eye advanced, thus opening the angle with the front of the thorax on that side. Measurement of the relative tilt of the body

and twist of the head showed that when the body tilted 30° from the vertical the sagittal plane of the head inclined 50°, with a body tilt of 45° the head was inclined 75°, while in extreme cases, with very bright illumination and over reactive flies, the body frequently tilted as much as 55° or 60°, sometimes even more, and the sagittal plane of the head was horizontal.

The same postural asymmetry may be produced by simply focusing onto the left eye of a normal robber fly a beam of light from the objective of the optical system of a string galvanometer; thus the right eye is relatively dark, there is an unequal photochemical reaction in the two eyes, and asymmetry in the tonus of the muscles of the two sides of the body is produced just as it is by producing the unequal photochemical change by blackening one eye.

In general it may be stated that all butterflies, including yellows, whites, meadow-browns, coppers, skippers, and fritillaries, react in the same way. They show abnormal postures when quiet, and circle toward the unblackened eye both when walking or flying. *Circionis alope*, *Vanessa huntera*, and *Argynnis aphrodite* are good types for illustration. When resting, the wings are held together above the back and show the tilt of the body in a striking way when one eye is blackened. The average body tilt in the bright light of the laboratory was 40° as shown in Fig. 4. This figure also shows the rotation of the head by the fact that the antennæ are displaced toward the seeing eye, often both being carried to this side beyond the sagittal plane of the body; in this feature there may also be some contraction of the muscles of the antennæ, which in extreme cases carries the lower antenna parallel with, or even touching the horizontal surface on which the butterfly is resting. *Vanessa* often holds its wings spread horizontally; this is the normal position of the wings of moths; and in these cases the wing on the side of the blackened eye is elevated while the other wing is depressed and touches the surface on which the animal rests. This asymmetrical condition of tonus of the wing muscles is maintained both when resting and when walking and it is probably the cause of the circus motions when flying. The tonus of the muscles of the anterior pair of walking legs of butterflies is so altered as to produce the asymmetrical postural conditions described for *Proctacanthus*, and to an exaggerated degree. The changes in the posterior legs are not so clear.

Most of the commoner flies (*Musca*, *Dexia*) show reactions, when one eye is blackened, which are completely in accord with those shown by *Proctacanthus* and butterflies, and the general description already given will suffice, but the banded-eyed fly or "green-eye," *Tabanus*, is the most typical and sensitive in its responses. All the postural changes already described are well exhibited by this fly, especially the lateral flexion of the broad thin head. After blackening one of its eyes the circus motions toward the unblackened eye are made in circles of very narrow diameter, so that this fly is well adapted to the study of effects of the intensity of light, to turntable experiments, and incidentally to the study of nystactic movements of the head,

FIG. 4. Butterfly seen from the front. Shows the tilting of the body and rotation of the head toward its left after blackening the right eye.

which occur in this insect when it is rotated in both directions, even after the eye has been blackened. The nystactic movements are so easily elicited in this fly, and some others observed by the writer, that they are produced by its own spontaneous movements to either side. The drone fly, *Eristalis*, also shows marked changes of muscle tonus and forced movements due to differences in the illumination of the eyes. To produce the typical results of blackening one eye as seen in other forms, it is necessary, in this insect, also to blacken the inner half of the other eye.[22] When this is done *Eristalis* shows

[22] Several forms did not exhibit typical results after blackening one eye; for example, the vespidæ and dragon flies. *Calliphora* also is atypical in its reactions, and *Eristalis* seemed to fall into the same group. It was found on *Eristalis* that

extreme tilting of the body toward the side of the uncovered eye surface. All the legs on this side are flexed until hidden under the body and the anterior leg is adducted far toward the side on which the black has been applied and may even be visible beyond the body on that side. In preening the abdominal segments with the posterior legs, the leg on the blackened side rubs only the lateral and dorsal aspects while on the side of the functioning eye the preening is confined to the lateral and ventral aspects of the abdomen.

The fruit fly, *Drosophila*, owing to its precise heliotropic reactions would be material of choice for these studies. When one eye is brilliantly illuminated, these flies show the typical flexion of the legs on that side. The blackening of the eye is a time-consuming procedure which offers practical difficulties due to the diminutive size; one cannot be certain that injury has not been inflicted, nor that complete blackening is accurately accomplished. Nature has performed the experiment for us however. Among the types of *Drosophilæ* raised by Professor T. H. Morgan, is a type possessing one normal red eye, the other eye being white, and blind or defective. Professor Morgan called my attention to the fact that these flies move in circles toward the normal eye. He gave me the opportunity, in one instance, of convincing myself that light acting on these one-eyed forms produces the same effects as on normal flies with one eye blackened. More recently I have studied many of these flies and found that the typical asymmetry in the position of the legs is characteristic, the legs on the side of the normal eye being flexed and even concealed beneath the body, while those on the side of the defective eye were extended. The flies moved in circles from the time they were hatched until they died; they never learned to correct the abnormal physiological condition of the musculature.

after blackening one eye the typical results were obtained, in exaggerated form, by blackening the inner half of the other eye. The writer has been led to the belief that in most of these forms each eye can function like both eyes of other forms. Each eye controls the muscle tonus of symmetrical groups of muscles on *both* sides of the body. Physiologically speaking these forms are *bilaterally cyclopic*. This possibility should be borne in mind when working with forms in which it is found that blackening of one eye does not produce the expected results; thereby one of the stumbling blocks to agreement on the heliotropic mechanism will be removed.

The fact that flies which have always been blind in one eye show the same changes in the muscle tonus and the same motor reactions as other insects with one eye blackened, eliminates all idea of "avoiding reactions." The further fact that the behavior of the insects in the above experiments, and in those to be described, is due to forced motions dependent upon the conditions of muscle tonus which exist in the *resting* animals, also disposes of any relation to "trial and error."

We may summarize the results of the above experiments by stating that the tonus of the muscles depends upon the illumination of the eyes. The muscle groups affected by the right eye are different from those affected by the left eye, thus a difference in illumination of the two eyes, whether induced by illuminating one or blackening the other, produces an asymmetry when the insects are at rest. The conditions of muscle tonus thus produced are such that if the animals move, they are forced toward the side of the greater illumination. As previously pointed out,[1] the mechanism of this forced motion is the same as that in the movement of animals in which the tonus of the muscles has been produced by other means, such as passing the electric current through the body as described by Loeb and Maxwell[23] and by Loeb and Garrey.[24]

Similar tonus changes and forced motions can also be produced by ablation or defects in the internal ear of vertebrates, so that as an organ of muscle tonus, the eye of insects is comparable to the otic labyrinth of vertebrates. In elaborating this conception we shall show that there are very intimate functional analogies between the eye of the insect and the vertebrate ear, which emphasize the similarity of this relationship to muscle tonus; thus the tonus of specific groups of muscles is controlled by definite areas on the insects' eyes much as they are in vertebrates by different parts of the otic labyrinth. The following experiments will make this clear.

Effect of Blackening Part of One Eye.—This procedure has the effect, although to a lesser degree, of blackening the whole eye; more accurately, it has the effect of decreasing the illumination in that eye. The extent of the tonus changes in the muscles is, roughly speaking,

[23] Loeb, J., and Maxwell, S. S., *Arch. ges. Physiol.*, 1896, lxiii, 121.
[24] Loeb, J., and Garrey, W. E., *Arch. ges. Physiol.*, 1897, lxv, 41.

proportional to the area blackened. Thus a dot of black centrally located on one eye may produce no appreciable effect upon the position of the legs of *Proctacanthus*, although by increasing the area circumferentially until half of the eye area is blackened, postural changes are produced. But even before enough of the eye has been blackened to show the resting postural changes, circus motions toward the normal eye take place.

While it is true that this result attends blackening of an equal area on any part of the eye, there is nevertheless a marked difference in the relation of different parts of the eye to the different muscles; for example, the radius of the circus movement is less when the outer half of one eye is blackened than when the inner half is thus treated. This was put to a simple test. The outer half of the right eye was blackened and the robber fly circled to the left; the inner half of the left eye was now blackened but the fly still circled to the left, although in a much larger circle than formerly, showing only a partial neutralization of the effect. By extending the line of blackening farther out on the left eye or decreasing the area of black on the outer side of the right eye, exact neutralization could be obtained, and by continuing this process *Proctacanthus* could be made to circle to the right. The reversal of the direction of circling is thus brought about in this fly by simply altering the relative amount of light entering the two eyes although the space relation of the light field to the dark field does not change. This reaction is wholly incompatible with the idea of an "avoiding reaction."

An experiment, performed on *Eristalis*, gave results confirming the contention that in this form, each eye controls the muscles of both sides of the body. Blackening the outer half of one eye may not produce a strong tendency to circle to the opposite side. The additional blackening of the inner half of the other eye, so that corresponding visual fields of both eyes are obliterated, produces marked circus motions—much more marked than result after blackening all of one eye. Still more marked is the effect of blackening all of one eye and the inner half of the other; the circles made by *Eristalis* then have a minimal diameter, the flies often simply rotating about the vertical axis of the body if the light is intense. The explanation of this phenomenon has already been considered.

The upper and lower parts of the eye show much more clearly the control of different groups of muscles by different areas of the eye. Experiments illustrating this were described by Axenfeld[25] who states that when the upper parts of the eyes of flies were blackened the head was elevated, and that it was depressed when the lower parts were blinded. Holmes[10,18] depicts marked postural changes when the eyes of *Ranatra* are similarly blackened, or illuminated from above or below. The results of our experiments on *Proctacanthus* are most striking and suggested to the writer the similarity to those produced by defects of the superior and posterior semicircular canals of vertebrates.

Effect of Blackening Lower Halves of Both Eyes.—The effects of blackening the lower halves of both the eyes of *Proctacanthus* are

FIG. 5. Robber fly with the lower halves of both eyes blackened. Body in opisthotonus, head up, front legs extended.

shown in Fig. 5. The posture is a striking one. The anterior and middle pair of legs are in extreme extension and pushed far forward. The anterior pair may be poised in the air. The anterior end of the insect is lifted far up and back from the surface of the table, and the head is tipped far back against the thorax so that the proboscis projects directly forward. The abdomen is concave dorsally in opisthotonus, and its terminal segments are pressed against the table. The wings rest on the abdomen or to the side of it.

When walking, these robber flies progress but slowly, and give the impression of climbing up into the air; they often poise resting on the depressed wings, tip of the tail, and posterior legs. The tendency to fly is pronounced and at the slightest disturbance these flies soar

[25] Axenfeld, *Arch. ital. biol.*, 1899, xxxi, 370.

upward and backward striking the cover of a confining glass dish or looping the loop backward. Upon alighting they fall on their backs and right themselves only by turning a backward somersault. If the lower parts of the eyes are unequally blackened, there is a combination of these effects with the tendency to circle toward the eye with more surface exposed to the light.

Effect of Blackening Upper Halves of the Eyes.—The resting attitude assumed by *Proctacanthus* with the upper halves of both eyes blackened is exactly the opposite of that just described, as may be noted in Fig. 6. The body of the insect is in emprosthotonus. The abdomen is concave ventrally and there is a considerable angle between the wings and the dorsum of the abdominal segments, due to the ventral

FIG. 6. Robber fly with the upper halves of both eyes blackened. Body in emprosthotonus, head down, anterior legs flexed.

flexion of the thorax. The head too is bent down with the crown forward and the frontal aspect pressed toward the table. The anterior legs are flexed, while the posterior legs by a combination of flexion and extension of different segments, tilt the thorax upward and forward so that in some cases the robber fly literally stands on its head. Such an animal when it creeps forward strikes the head against the slightest obstacle and is precipitated in a forward somersault. In attempted flight it is not able to lift the body and crowds forward and down against the surface of the table, while if thrown into the air, it is precipitated downward in a forward somersaulting flight to the floor, where it lands on its back. In this position the fly is almost helpless, being able to right itself only if its struggles make it favorable to turn sideways; otherwise its futile efforts result in a ventral flexion of the

body in which the anterior and posterior ends of the insect meet in a ring.

Effect of Blackening Upper Half of One Eye and Lower Half of Other Eye.—By blackening the upper half of one eye and the lower half of the other eye of *Proctacanthus*, one obtains a combination of the effects described in the preceding experiments. This procedure emphasizes the fact that different parts of the eye control the tonus of entirely different groups of muscles on the two sides of the body. The resulting tonus changes produce a most bizarre type of asymmetry

FIG. 7. Robber fly with the upper part of the left eye and the lower part of the right eye blackened. Body tilted to the right and twisted on its long axis. Head rotated to the right. Left anterior leg and right posterior leg extended. Right anterior leg and left posterior leg flexed. Moves in circles to the right.

in the position of the legs at rest, and in the character of the movements (Fig. 7). Thus with the lower half of the right eye and the upper half of the left eye blackened, there is flexion of the anterior leg of the right side and extension of its mate on the left side. There is flexion of the posterior leg on the left side and extension of the right posterior leg. The whole body is thus twisted on its long axis. The head is depressed and rotated down on the right side, that is toward the blackened area of the eye of that side, up on the left side so that the sagittal plane of head is horizontal. The tail end is twisted down on the left side.

When these insects move they do so in a most clumsy incoordinated manner, always in small circles to the right in spite of the fact that equal areas of the two eyes are blinded, indicating again the fact that different areas of the eyes control the tonus of entirely different groups of muscles. Flying movements are absolutely disorganized. The wings have no lifting power and the fly simply spins about a vertical axis, never leaving the surface of the table. If thrown into the air, the body may rotate on its long axis and gyrate in so incoordinated a way that schematic analysis is impossible. One is reminded again of asymmetrical lesions in the labyrinths of the vertebrate ears.

Effect of Symmetrical Blackening of Eyes.—Symmetrical blackening of the eyes, both outer halves or both inner halves for example, does not result in any asymmetry of posture either of the body, legs, or wings of *Proctacanthus*, provided equal areas are blackened on the two sides. There is weakening of some groups of muscles, but, the two sides being symmetrically affected, there are no forced motions either to the right or left.

Comparison of the Insect's Eye with the Otic Labyrinth of Vertebrates.—The experimental results afford irrefutable proof that the tonus of the muscles, their excitability, and force of contraction, and thus the basal conditions for coordinated movement, are dependent upon the effects of light upon the eyes of heliotropic insects. The otic labyrinth subserves these functions in vertebrates. In the latter, entirely different groups of muscles are affected by different parts of the labyrinth, and the analogy to the insect's eye is made complete by our demonstration that different parts of the eye control the tonus of different groups of muscles. Blackening the right eye affected very different groups of muscles from those affected by a similar treatment of the left eye; hence the circus motions were to the left or to the right respectively. The outer and the inner halves control different muscles. There are striking differences between the effects of blackening the upper and the lower halves of the eyes. There thus exists an *optical mosaic* in which the receptors are arranged in definite patterns which are mirrored in the groups of muscles involved in the effects of light and shade on these different areas.

It is possible even to trace, with a considerable degree of certainty, the path of the nerve impulses from the eyes to the different groups of

muscles. Such a detailed study is beside the purpose, and beyond the scope of this communication; a brief reference will suffice. Blackening one eye of positively heliotropic insects decreases the tonus and weakens the extensors of the opposite side, resulting in a flexion of the legs due to the unopposed action of the flexors. There is then at least a partial crossing of extensor nerve paths. The condition is similar to that demonstrated by operative methods on *Gelasimus pugnax* by Loeb and Maxwell.[26] The nerve paths between the eye and the flexors are not crossed; thus blackening one eye decreases the tonus and weakens the flexors on the same side and extension is unopposed. With the upper halves of the eyes blackened, the tonus of dorsal muscles is decreased and the extensors of the anterior legs are principally weakened with resulting flexion. Loss of function of the lower part of the eye weakens the flexors of the anterior legs, which become extended by the unopposed extensors, while the loss of tonus of the ventral body muscles results in opisthotonus. Other relations are easily made out.

Masked Conditions of Tonus.—The truest gauge of the physiological condition of a muscle is to be found in the character of its responses. The circus motions, which result from blackening one eye, are the result of asymmetrical conditions of the tonus of muscles of the two sides; these conditions exist in the resting muscle as is indicated by the resting postures. It often happens however that these resting postures are not in evidence, especially if the difference in illumination of the two eyes is not great, or in faint illumination after one eye has been blackened. Postures seen in one form may rarely if ever be evident in another; for example, the lateral bending of the abdomen of *Proctacanthus* often is not evident under ordinary illumination after one eye is covered. Activity however unmasks the true state of the muscle. Thus a sharp blow on the table, touching the insect with a bristle anywhere on the body, or a gentle blast of air delivered from any angle, elicits a general contraction of the body muscles without any progressive movement. In this response the expected postures are always assumed, at least momentarily; for example, a lateral bending of the abdomen of *Proctacanthus* always

[26] Loeb and Maxwell,[23] p. 134.

takes place toward the unblackened eye, and butterflies which may not lean toward the good eye, topple over toward that side if stimulated; they resist pressure against the wings from the side of the good eye but fall sideways if pushed or blown against from the side of the blackened eye. In alighting from flight such butterflies topple over toward the good eye as a result of the incoordination which results when the leg muscles are suddenly called into action. Such butterflies in righting themselves may spin about several times toward the functioning eye, although when at rest they may show no postural abnormality. These reactions demonstrate that there exists a real asymmetry in the physiological state of the muscles of the two sides, a weakness and absence of tonus which is unmasked only when a motor effort is made. We have here a perfect analogy to phenomena seen in vertebrates with certain labyrinthine abnormalities.

Asymmetrical Sensitiveness.—The maintenance of symmetrical equilibrium in the tonus and physiological state of the muscles of the two sides of the insects with which we worked, depends upon equivalent stimulation of both eyes. This is accomplished if the intensity of the illumination is the same, and the sensitiveness of the two eyes is equal. The effect of a stimulus varies directly with its intensity and also with the sensitiveness of the receptive tissue. It occurred to the writer that circus motions should result if one eye could be made more sensitive than the other; these should be in the direction of the more sensitive eye when both are equally illuminated. It was noted that *Proctacanthus* with one eye blackened shows the most pronounced postural changes and moves in circles of smallest diameter when brought from the dark room or during the early morning. The fact was established that exposure to light produces considerable fatigue of the eye and consequent weakening of the effect of light as noted in the progressive increase in the diameter of their circus movements, and that complete recovery of sensitiveness occurs after confinement in the dark. These facts suggested the expedient of simply removing the black from one eye after exposing the other to light for some hours. We were certain that the covered eye would be dark-adapted and relatively much more sensitive than the eye which had been exposed to light. Asphalt varnish hardens into a thin brittle scale and is easily cracked from the eye by a pair of fine pointed

forceps after a day or two. If the right eye had been covered, the *Proctacanthus* circled to the left, but immediately upon flaking off the covering and flooding the eye with light the direction of the circus motion was reversed and the insect circled to the right,—the side from which the black had been removed. The normal eye, owing to its previous exposure to light is fatigued; it has now become physiologically darkened although there has been no change in the illumination of that eye. The muscle tonus is affected by the greater sensitiveness of the right eye as if it were intensely illuminated. The effect may be produced by the removal of only a very small part of the black covering. The abnormal sensitiveness may persist for 2 hours or more but gradually wears off as the eye becomes light-adapted or fatigued, and the robber fly then behaves like a normal one. The experiment is a crucial one, demonstrating that the muscle tonus is proportional to the rate of photochemical reaction in the eye.

Effects of Intensity of Light upon Muscle Tonus.—Evidence has already been presented proving that muscle tonus is maintained by the action of light on the eyes and is lost in the dark, also evidence indicating that the tonus is proportional to the intensity of the illumination of the eyes. This can be demonstrated easily by the changes which result in the circus motions and postures of the insects upon varying the illumination after one eye has been blackened. A butterfly such as *Circionis alope* thus treated flew in circles with a diameter of 5 or 6 feet in the diffuse light of a room with windows on three sides, while in the sunlight the circles of flight were reduced to a diameter of 2 or 3 feet. Similarly when walking, the diameters of the circles were 2 or 3 feet when in diffuse light, but only a few inches in the sunlight. The degree to which resting butterflies leaned toward the good eye in diffuse light was distinctly increased by reflecting sunlight onto the eye from any direction. The bilateral asymmetry of the legs of *Proctacanthus* was much more marked in several individuals in the sunlight than when they were shaded, and the circles of movement had a much smaller diameter in the sunlight. *Tabanus* with one eye blackened, and *Eristalis*, with the additional blackening of the inner part of the other eye, lean far toward the seeing eye surface in sunlight and often simply circle about the tip of the posterior leg as a center, rarely in a circle with a diameter of more than 1 inch.

On the other hand, if these flies are shaded the diameter of the circles increases to 3 or 4 inches, while in the dim light of our dark room with open door, no tendency to circle was noticeable.

These effects are a function of the intensity of the illumination and are independent of all orienting effect of the light from a single source. Experiments devised to demonstrate this fact were performed by the author in 1912 and were described in lectures at Woods Hole that year.[27] The experimental insects were placed on the bottom of an illumination chamber made from a cylindrical dish, 20 inches in diameter and 12 inches deep, lined throughout with white blotting paper; the dish was covered with ground glass, and illuminated centrally from above by tungsten lights. Peep holes were provided for observation. With a 10 watt light, although one eye had been blackened, the insects moved about indifferently or at least had no difficulty leaving the floor and ascending the walls of the dish. With a 25 watt light they kept on the bottom; when a 100 watt light was used the circles were never over 6 or 8 inches in diameter, while a light of 400 watts caused a pivoting in one spot.

Results which lead to the same conclusions were obtained by partially excluding light from one eye by painting with collodion in ether-alcohol solution or with shellac in alcohol. The effects of a single layer of collodion were hardly noticeable. Successive layers, especially when milky, produced progressive narrowing of the circles of movement. Shellac had the same progressive effects until the result of total blinding was produced.

The results of these experiments justify our assertion that the muscle tonus varies directly with the intensity of the illumination, and that the asymmetry of the muscular tone, upon which circus motions depend, is determined by the difference in illumination of the two eyes. The same conclusions are reached by summing the effects of gravity with light, as described below.

Experiments with Heliotropic Insects Placed upon a Vertical Surface.—Butterflies, with normal eyes, and common flies walk directly up a

[27] Similar experiments have been described by Holmes and McGraw (*J. Animal Behavior*, 1913, iii, 367) who describe "orienting effects with a constant illumination." Dr. Minnich under the direction of Professor G. H. Parker has made quantitative determinations of circus motions under similar experimental conditions; we regret that they are not yet available for reference.

vertical surface or rest with the body axis in a vertical line. When one eye has been blackened, they no longer walk vertically but veer off at an angle toward the unblackened eye. There is an algebraic summing of the tendency to follow the vertical path and to circle toward the good eye which results in the oblique path. When at rest, the body axis preserves this obliquity. Several butterflies with one eye blackened were put upon an opaque, gray, vertical screen, so placed in the laboratory that one side received the direct light from the window while the other was shaded. On the shaded side the angular deviation of the path was 15° from the vertical. When the screen was reversed, the brighter illumination from the windows caused a deviation to a path 45° from the vertical, and when placed in the sunlight, the average path in which they walked was 75° from the vertical. In the latter instance the circling tendency was often noted, and the butterflies' path might deviate to the horizontal line; they then completed a sharp turn downward toward the good eye and swung completely around again, starting the vertical ascent, but at once began to swerve off at an angle. If the butterflies are placed upon a screen, the upper half of which is in the sunlight, the lower half shaded, one is surprised at the prompt increase in the angular deviation of the path as they cross the line from the shade into the sunlight. The flies, *Tabanus* and *Eristalis*, were unable to ascend a vertical screen when it was directly illuminated by bright window light but move in circles on it; in dim light they ascend obliquely.

Experiments on a Cylinder or Spindle.—Instead of a plane, vertical surface an upright cylinder or stick may be used. The position assumed by *Proctacanthus* (Fig. 8) is oblique to the vertical and shows the typical flexion and extension of the legs due to blackening one eye. When the spindle is placed in the center of the illumination chamber described above, or when light is reflected uniformly from all sides, the effects of variations in the intensity of illumination may be determined. All heliotropic insects, with one eye blackened, ascend such cylinders obliquely (Fig. 8), and the forced motion carries them up in a uniform spiral path. Instead of measuring the angular ascent, as on the plane, vertical screen, relative effects are determined by counting the numbers of turns required to ascend a given distance. The arrangement constitutes a crude photometer, for the brighter

the light, the greater the number of spiral circuits required to make the ascent. *Tabanus* and *Eristalis* are excellent flies for these experiments. They may be dealated or the wings glued together to prevent flying. It is convenient to place the flies inside a graduated glass cylinder to prevent escape. Surround this cylinder with a much larger one of white mat-paper, or blotting paper, and illuminate by light reflected from above. In one such experiment, which is quite typical, an *Eristalis* made the ascent of the inner wall of the graduate in two spirals, when illuminated by dim artificial light placed above the

FIG. 8. Position of a robber fly on a vertical spindle after blackening the right eye. It creeps up the spindle in a spiral path to the left.

cylinder in the dark room. In the laboratory, with the window shades down, the fly made four spiral turns. With the shades up, twelve spiral circuits were completed in making the ascent of 12 inches. When illuminated by sunlight, the fly simply pivoted on the wall of the cylinder and was entirely unable to make the ascent. In the latter instance the marked difference in the tonus of the muscles of the two sides, caused by the intense light, produced forced motions about the fly's dorsoventral axis.

If an opaque spindle is arranged vertically so that one side is in the bright light, the other in the shade, insects with one blackened eye

move in very different paths on the two sides. On the shaded side the spirals are parallel and the pitch is acute, but in the bright light of the other side the fly's path is more nearly horizontal; it may become horizontal and then the fly moves in a small circle downward and may thus be trapped on the bright side of the spindle. It resumes its upward path only if, when executing the smaller circles, it is carried to the shaded side of the spindle. The different character of the paths on the two sides is only the expression of forced movements due to the different conditions of muscle tonus in light of different intensities. The facts are significant in that they explain the movement of positively heliotropic insects to a light even when one eye is blackened. They do so, as the records of many observers attest, in a succession of large and small circles. In circling, the good eye is successively illuminated by, or shaded from, the luminous source. The large arcs are executed when the good eye is shaded, and the smaller arc is traversed when the good eye is coming into bright light. The effect is due only to the effects of the varying illumination upon the muscle tonus. The movements are "forced motions"—thus vanishes the mystery of "trial and error" in these instances. The mechanism of the "attraction" is the same as that in true heliotropism of flies with both eyes normal.

A study of the behavior of heliotropic insects on a turntable has yielded results confirming the conclusions drawn from the preceding experiments. These tests included observations on positively heliotropic flies, butterflies, and beetles, slow walking forms being best adapted to the end; *Tabanus* and *Eristalis* gave excellent results. Loeb[2] (1890) first described the fact that flies when rotated on a turntable, showed compensatory circus motions in a direction opposite to that of rotation. Lyon[8] showed that these disappear when the eyes are blackened, and Rádl[12] believed them to be the result of a visual fixation. These reactions, as well as the nystactic movements of the head of insects, are so suggestive of those of vertebrates, where they are due to the internal ear, that they strongly emphasize the fact that the muscle tonus of the insecta is controlled by reflexes from the eyes.

Experiments on the Turntable.—A normal fly (*Tabanus* for example) was placed in a cylinder at the center of a turntable, illuminated

equally on all sides. The ascent of the wall was made by the normal fly in a vertical line when the cylinder was stationary. When the cylinder was slowly rotated the fly circled in the opposite direction as if to maintain a vertical line of ascent; the result of the rotation however is to turn the body axis to an angle with the vertical, and the fly traverses a spiral path on the wall of the revolving cylinder. Increasing the speed of rotation (within limits) increases the number of spirals and may even cause the fly to walk horizontally or simply circle on the wall, ascent thus being rendered impossible. When this experiment is performed after blackening one eye, the fly ascends the stationary cylinder in a spiral path as described previously. Slow rotation which carries the fly toward the side of the blackened eye intensifies this forced motion, the number of spirals is increased, or the vertical component of the path is entirely nullified with a much slower rotation than in the normal fly. On the other hand, if the rotation is toward the unblackened eye, the tendency to circle in the opposite direction, which was noted in the normal fly, is still evident in the fly with the blackened eye, for it neutralizes the circus motions in part or wholly depending upon the speed of rotation. An appropriate speed may be found at which the fly no longer moves in a spiral path but ascends a vertical line on the wall of the cylinder. Faster rotation will cause ascent in a spiral in the opposite direction—a forced circus motion toward the blackened eye.

The intensity of the illumination has a decided effect on the turntable reactions of these flies. It requires a greater speed of rotation in the bright light to cause the change from a spiral to a vertical path, and conversely, if the spiral path has been changed to a vertical one by rotation toward the unblackened eye, an increase in the illumination will restore motion in a spiral path toward the good eye. This is of course due only to a reestablishment of a difference in the muscle tonus of the two sides by increase in the illumination of the unblackened eye.

Concordant results are obtained on the horizontal surface of a turntable. A fly, blinded in one eye, circles in a given radius; this radius is decreased and the fly simply turns about a vertical axis in one spot if rotated toward the blind eye. The circles are widened, or circling in the opposite direction results by rotating toward the good eye

depending upon the speed employed. The brighter the light, the easier it is to narrow the circles by rotation toward the blackened eye and the more difficult it is to neutralize the forced motion by rotation toward the normal eye. Possibly the similar behavior, on the turntable, of flies from which Loeb had removed one-half of the brain, was due to the fact that the light stimulus from one side was lacking.

In conclusion it may be stated that the condition of muscle tonus produced by blackening the eyes was not recovered from in the weeks during which many robber flies and butterflies were kept in the laboratory; as a matter of fact, the asymmetry of the muscles gradually became more pronounced and was fixed after death by the onset of rigor mortis.

SUMMARY.

The tonus of the muscles of heliotropic insects is due chiefly to the action of light; it is markedly decreased in the dark. Each eye controls the tonus of a different group of muscles on both sides of the body. Different areas of each eye likewise are related to the tonus of different muscle groups, and the relationship is entirely analogous to that of the otic labyrinth of vertebrates. Asymmetrical conditions of muscle tension are produced by any procedure which establishes an unequal photochemical reaction in the two eyes, by difference in illumination, by partial or complete blackening of one eye, or establishing unequal sensitiveness in the two eyes. The unbalanced condition of muscle tonus expresses itself in unusual postures of the resting insects, and in movements in forced paths—circus motions when one eye has been blackened. These reactions vary directly with the intensity of the illumination, as shown not only by the variation in diameters of the circles, but also by the reactions of the insects on vertical surfaces and on the turntable.

The relation of the results of these experiments to the problem of heliotropic orientation is too obvious to require detailed discussion, which could only lead to a repetition of the description of the mechanism of heliotropism which Loeb has so clearly expounded. The experiments are so completely in accordance with Loeb's muscle tension theory of heliotropism, that they are tantamount to a complete proof of it.

LUTEAR CELLS AND HEN-FEATHERING.

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(Received for publication, August 7, 1918.)

In nearly all breeds of poultry the dorsal plumage of the male differs from that of the female in length of certain feathers, in structural features, and in most breeds in color also. In the race of Sebrights, on the other hand, the male is feathered like the female. He is said to be hen-feathered. There are other breeds, such as the Campines and Hamburghs, in which both cock-feathered and hen-feathered adult males are known.

It has recently been shown by Boring and Pearl[1] that there are groups of cells in the ovary of the hen (Fig. 1) that collect in the follicles after the egg is set free, and produce there a yellow pigment that reacts chemically in the same way as does the lutear pigment of the corpus luteum of the mammal. They call these cells lutear cells.

Boring and Pearl have also shown that the lutear cells are absent in the testes of adult male fowls.

It has been convincingly demonstrated by Goodale,[2] both for ducks and fowls, that extirpation of the ovary leads to the assumption of the full male plumage by the female. Whether the germinal material, or the connective tissue of the stroma of the ovary, or the lutear cells are responsible for the condition of the plumage of the female could not be determined by ovariotomy alone. If, however, any element should be found in the testes of the hen-feathered Sebright that was absent from other cock-feathered breeds, and like any elements peculiar to the female, then it would appear highly probable that these elements

[1] Boring, A. M., and Pearl, R., *Anat. Rec.*, 1917, xiii, 253; Pearl and Boring, *Am. J. Anat.*, 1918, xxiii, 1.

[2] Goodale, H. D., *Biol. Bull.*, 1910–11, xx, 35; *Am. Nat.*, 1913, xlvii, 159; *J. Exp. Zool.*, 1916, xx, 421.

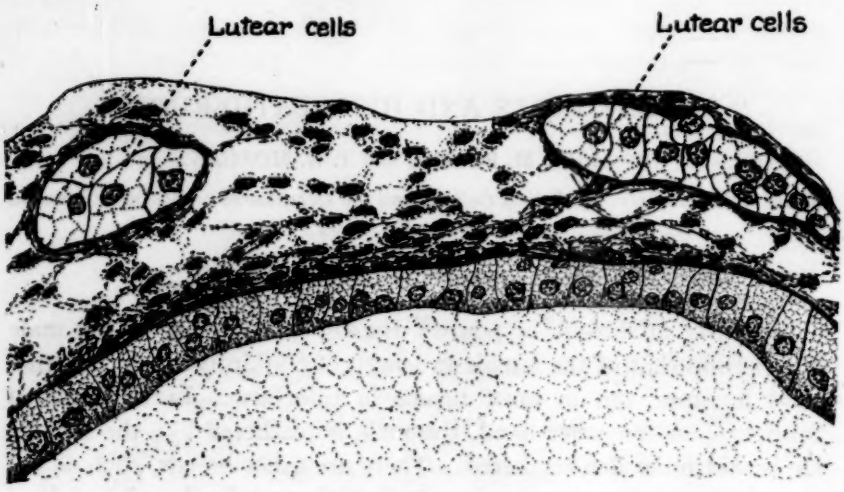

FIG. 1. Two groups of lutear cells in the theca of the follicle of the hen.

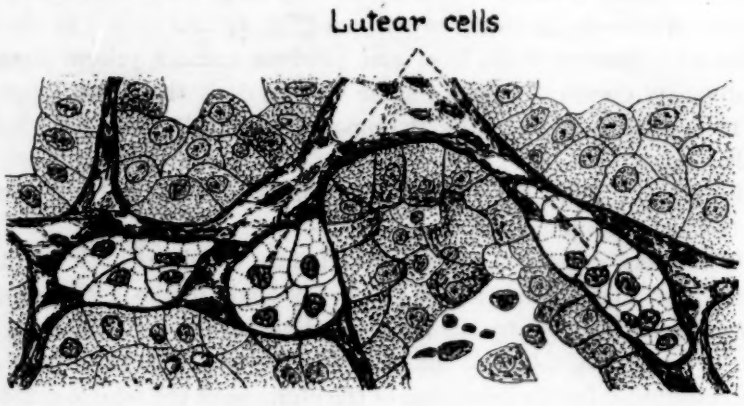

FIG. 2. Three groups of lutear cells in the connective tissue between the seminal tubules of the Sebright male.

are responsible for hen-feathering in the female as well as in the male Sebright.

A histological examination of the testis of a Sebright has shown that it contains groups of lutear cells (Fig. 2) identical in appearance with those in the ovary of the hen. It seems practically certain that these

are the cells whose secretion suppresses in the hen and in the Sebright male the characteristic cock-feathering.

In support of this conclusion it may be pointed out that one of us has recently shown that complete removal of the testes from the male Sebright causes him to assume the plumage of the ordinary cock.[3] The result is the same as removal of the ovary from the hen (Good-

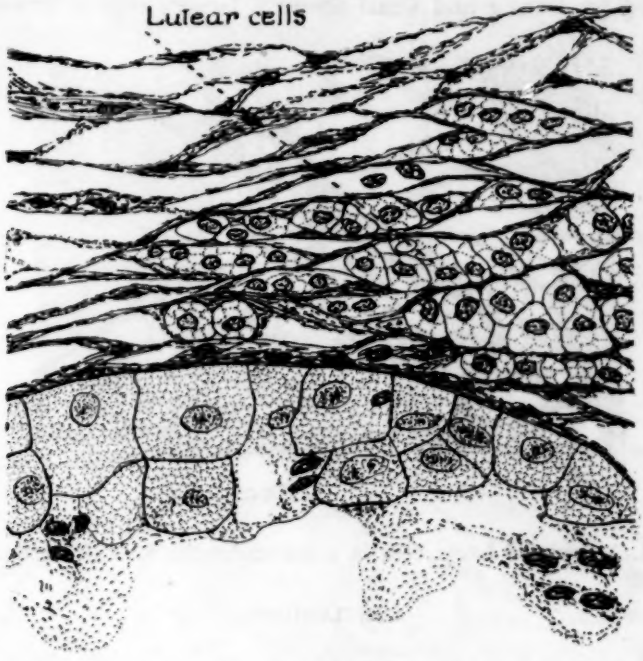

FIG. 3. Several groups of lutear cells in the regenerated testis of a Sebright male.

ale), and theoretically due in both cases to the removal of the lutear cells. The complete demonstration of the above conclusion should be obtained by implanting in an ovariotomized hen pieces of the testes of a Sebright male, producing thereby the same effect as that produced by the presence of her own ovary.

[3] Castration of the ordinary cock does not change the character of his plumage.

In Fig. 1, two groups of lutear cells, in the wall of an egg follicle of a hen, are shown. In Fig. 2 three groups of lutear cells are seen between the tubules of the testis of a male Sebright. In Fig. 3 lutear cells in the regenerated testis of a Sebright male are shown. The bird had been castrated but, since it did not change the character of its plumage, after 6 months it was opened and this piece of testis was found and removed. In Fig. 4 a piece of the testis of a hermaphrodite bird described by Boring and Pearl shows a large group of lutear cells.

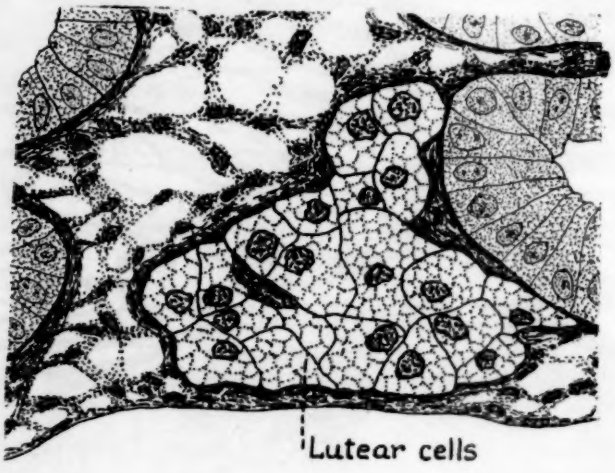

FIG. 4. A group of lutear cells in a hermaphrodite fowl (from Boring and Pearl).

CONCLUSIONS.

The experimental evidence had made clear that some substance is produced in the testis of the male Sebright that suppresses in him the development of the secondary sexual plumage of the cock of his species. The detection in his testis of lutear cells like those in hens makes the conclusion highly probable that it is these cells that cause the suppression of cock-feathering in both the Sebright male and in hens of all fowls. Genetic work by Morgan[4] had shown that one or two Mendelian factor-differences are responsible for hen-feathering

[4] Morgan, T. H., *Am. Nat.*, 1917, li, 513; *Proc. Soc. Exp. Biol. and Med.*, 1915–16, xiii, 31.

in the Sebright. These factor-differences produce their effects through the testes. The presence of these genetic factors, we now see, causes the testes of the Sebright to produce a kind of secretory cell that is ordinarily only produced in the female, or possibly to a slight extent in young males (Boring), or in numbers insufficient to suppress the male plumage in the testes of some ordinary cock birds (Reeves[5]).

[5]Reeves, T. B., *Anat. Rec.*, 1915, ix, 383.